JUSTUS-LIEBIG UNIVERSITY GIESSEN
AND
HOCHSCHULE GEISENHEIM UNIVERSITY

THE ALLERGIC POTENTIAL ARISING FROM PROTEINOUS WINE FINING AGENTS OF MILK AND CHICKEN EGG ALBUMEN

by

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M.Sc. Montpellier SupAgro, 2009

Submitted in partial fulfilment of the

requirements for the degree of

Dr. agr.

2014

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Declaration

"I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation.
I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me.
At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice"

The 20th of March, 2014

Manuella Webber-Witt

Note of thanks

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O amor de Deus repousa em mim.

"The eternal mystery of the world is its comprehensibility"
-Albert Einstein

"Live as if you were to die tomorrow.
Learn as if you were to live forever"
-Mahatma Gandhi

"It always seems impossible until it's done"
-Nelson Mandela

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List of abbreviations

[]	Concentration
BRC	British Retail Consortium
CAP	Common Agricultural Policy
CaT	Calcium hydrogen tartrate
CD	Celiac disease
CFU	Colony-forming unit
CIELAB	Commission Internationale de l'eclairage (L*a* b*: coordinates)
CO2	Carbon dioxide
CV/VC	Coefficient of variation
DBPCFC	Double-blind placebo-controlled food challenge
EC	European commission
EDI (CH)	Eidgenössisches Departement des Innern/Federal Department of Home Affairs (Switzerland)
EFSA	European Food Safety Authority
EK	EK – filtration: sterile filtration
EU	European Union
FCR	Folin-Ciocalteu-Reagent
FDA	Food and Drug Administration
FEI	Forschungskreis der Ernährungsindustrie - Research Association of the German Food Industry
FPIES	Food protein induced enterocolitis syndrome
FSANZ	Food Standards Australia New Zealand
GFSI	Global Food Safety Initiative
GMP	Good Manufacturing Practices
GRP	Grape-Reaction-Product
GWL	Guide to Wine Law
HPLC	High-performance liquid chromatography
IFS	International Food Standard
IgE	Immunoglobulin E
ISO	International Organization for Standardization
kDa	Kilodalton
kg	Kilogram
KHT	Potassium hydrogen tartrate/Potassium bitartrate
l/L	Litre
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of detection
(L)LOQ	(Lower) Limit of quantification
LTP	Lipid-transfer protein
µS	Micro Siemens
mg	Milligram
MLF	Malolatic Fermentation

mm	Millimetre
MT	Mueller-Thurgau
n.d	Not detectable
NDA	Nutrition and Allergies
nm	Nanometre
NOAEL	No-observed-adverse-effect level
OFC	Oral food challenge
OIV	International Organisation of Vine and Wine
PN	Pinot Noir
PC	Personal Computer
pcm	Per cent mille
PCR	Polymerase chain reaction
pI	Isoelectric point
ppb	Part(s) per billion
ppm	Part(s) per million
PVPP	Polyvinylpolypyrrolidone
QS	Quality System
SBPC	Single-blind placebo-controlled
SD	Standard deviation
SDS-Page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard Operating Procedure
SPT	Skin Prick Test
ssIgE	Serum specific IgE
TEAC	Trolox®-Equivalent-Antioxidative-Capacity
TRFA	Total reflection fluorescence analysis
TTB	Alcohol and Tobacco Tax and Trade Bureau
VITAL	Voluntary Incidental Trace Allergen Labelling
WAO	World Allergy Organization
λ	Lambda

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1 Summary

This study investigated whether technological procedures could be used to avoid allergic residues remaining in wine, after the use of fining agents containing milk or egg protein. Fining materials used during the wine making process when derived from milk or egg may present the possibility to trigger an allergic reaction. Therefore these substances should be declared on the wine label if present in the final product, depending on the legislation of each country. Label information enables allergic consumers to avoid food and beverages that may trigger allergic symptoms. As there is no standard for the sequence of winemaking practices a fining trial was conducted to study the influence of various filtration and further methods normally used in the wine industry and its efficiency on reducing this possible allergens, even in a worst case scenario. More than nine different German wines, red and whites from different years were used on this study. The methods used to detect the residues were different ELISA assays and *in vivo* tests were evaluated with allergic patients, prick skin test and Double-blind, placebo-controlled food challenge (DBPCFC) with fined wines, where none of the patients tested reacted allergically.

Casein is removed from both red and white wines to not detectable levels by all methods of filtration used in this work. Centrifugation alone might let some residues behind as well as flash-pasteurisation. Nevertheless an additional sterile filtration after flash-pasteurisation or centrifugation decreases casein proteins to no longer being detectable.

For whey protein only a bentonite treatment with successive sterile filtration was found to be competent to reliably reduce the amount of detectable protein below the detection limit of the assay at both concentration levels in both red and white wine used in this study.

Albumin residues are present when wine is fined but not filtered, especially white wines, most filtration methods used in this work are capable to bring the residues to no longer being detectable when a normal dosage of fining agent is applied. Flash-Pasteurisation is not advised for wines that have been previously fined with egg white, since there is an increase on the residues after heating practice, when ELISA assay is applied as detection method. Lysozymes stay present in the wine in most cases of this study, in reds and in whites. If a bentonite treatment takes place after the fining, in the legal limits of up to 500 ppm, followed by sterile filtration with 0.45µm pad filter, no residues are detected.

Lysozyme reacts with metatartaric acid and with carboxymethyl-cellulose and precipitates, but not completely. Reaction with red wine phenols lead to precipitation, the higher the total phenol content the greater was the enzyme precipitation, here

again the precipitation itself was not enough to diminish the lysozyme amount under 0.25 ppm.
In conclusion, if wines are fined with low amount of fining agents and properly filtered, they are likely to have no detectable residues. However if a wine is not filtered and casein, whey protein and egg albumin or lysozyme has been applied it should be declared on the label.

2 Introduction

A food allergy is an immune system response to a food that the body mistakenly believes is harmful. Existing figures show that probably <2% of the adult population suffers from a food allergy. Allergies may constitute a danger to the health of those concerned and a severe allergic reaction can even lead to death (Sampson, 2004).

The European Union is the world-leading producer of wine, a traditional alcoholic beverage; wine. The EU regulates among others the labelling of wine, especially with the intention of protecting consumers. Therefore label information enables allergic consumers to avoid substances that may trigger allergic symptoms (Sampson, 2004). At the present moment there are only a small number of studies regarding the residues, the quantity and the risks associated with them that these fining materials may leave behind in the wine, especially concerning possible implications for allergic consumers.

The purposes of fining may include clarification, stabilization or improvement of taste and a wine may be fined by adding diverse oenological agents, depending on the wine and on the aim of fining (Troost, 1988; Christmann and Freund, 2004). There is no standard for the sequence of winemaking practices. It is for the winemaker, in accordance with each country's law, to select the quantity of fining agents that are to be applied. The winemaker also decides subsequent procedures that the wine will pass through after the fining itself, such as filtration. Fining materials that are derived from milk and egg products, which can possibly trigger an allergic reaction, are sometimes used during the wine making process. These substances are included in the Directive 2003/89/EC and Regulation 2010/1266/EC, and therefore should be declared on the label of wine, if present in the final product.

These studies were carried out with the purpose of determining whether residues of these specific substances remain in the wine and the possibility of quantifying them. Recent European law; Regulation 2010/1266/EC, permitted the labelling and marketing of wines without declaring these substances up to the 30th of June 2012 or until these stocks are exhausted (Teissedre, 2011). Any wine produced or bottled after this date must declare on the label if it contains 0.25 ppm or greater of egg or milk allergens (EU Regulation 579/2012).

This study also researched whether technological procedures could be used to avoid allergic residues remaining in wine, after the use of fining agents containing milk or

egg. These techniques developed in the Oenology Department of Geisenheim include using different filtration methods to remove allergenic fining materials, and the use of alternative fining agents that are not required to be labelled.

This study is a collaboration between three project partners; University of Hamburg, Department of Chemistry, Institute of Food Chemistry – responsible for methods of detection, the Munich Technical University - Department of Dermatology and Allergology – responsible for allergic testes with allergic patients and the institute of oenology of Geisenheim University – responsible for all wine making process and fining.

The research project (AiF 16330 N) was funded under the program to promote Industrial Joint Research (IGF) of the Federal Ministry and Technology (via AiF) by Research Association of the German Food Industry (FEI).

3 Literature survey

3.1 Labelling in Europe

Recent years show an increased number of subjects suffering allergic reactions and intolerances coming from food ingredients. For this reason on the 25th of November 2003 the Directive 2003/89/EC of the European Parliament entered into force as well as the Council of 10th November 2003 amending Directive 2000/13/EC regarding information of ingredients that are contained in foods. 'Ingredient' shall mean any substance, including additives, used in the manufacture or preparation of a foodstuff and still present in the finished product, even if in altered form. The European Commission has declared in this Directive 2003/89/EC – the "Allergen Labelling Directive", the 14 most frequent potential allergens as presented in Table 1. These allergens should be declared clearly on food label if they are contained directly in the food as a single ingredient or have been used as a compound or ingredients used throughout the food production. Some of these listed ingredients are used during wine production (EU Directives, 2000 and 2003).

Currently there is no known therapy for food allergies. An option for all relevant patients is the avoidance of the respective allergen. The Scientific Committee on Food has stated that the incidence of food allergies may affect the lives of many people, causing conditions ranging from very mild to potentially fatal. These directives should therefore help consumers to be informed about possible allergens in food. Every ingredient of Table 1 that is likely to cause adverse reactions in susceptible individuals should be declared on the label independent of the amount in which it is present. The list of allergenic substances should include those foodstuffs, ingredients and other substances recognized as causing hypersensitivity. In order to provide all consumers with better information and to protect the health of certain consumers, it should be made obligatory to include in the list of ingredients all ingredients and other substances present in the foodstuff. In the case of alcoholic beverages, the regulations should state the same (EU Directives, 2003).

Table 1 Update version of Annex III a of EU Directive 2003/89/EC

1.	Cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, kamut or their hybridised strains) and products thereof, except: (a) wheat-based glucose syrups including dextrose; (b) wheat-based maltodextrins; (c) glucose syrups based on barley; (d) cereals used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages.
2.	Crustaceans and products thereof.
3.	**Eggs and products thereof.**
4.	Fish and products thereof, except: (a) fish gelatine used as carrier for vitamin or carotenoid preparations; (b) fish-gelatine or Isinglass used as fining agent in beer and wine.
5.	Peanuts and products thereof.
6.	Soybeans and products thereof, except:
	(a) fully refined soybean oil and fat; (b) natural mixed tocopherols (E306), natural D-alpha tocopherol, natural D-alpha tocopherol acetate, natural D-alpha tocopherol succinate from soybean sources; (c) vegetable oils derived phytosterols and phytosterol esters from soybean sources; (d) plant-stanol ester produced from vegetable oil sterols from soybean sources.
7.	**Milk and products thereof (including lactose), except:** **(a) whey used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages;** **(b) lactitol.**
8.	Nuts, i.e. almonds (Amygdaluscommunis L.), hazelnuts (Corylusavellana), walnuts (Juglansregia), cashews (Anacardiumoccidentale), pecan nuts (Caryaillinoiesis (Wangenh) K. Koch), Brazil nuts (Bertholletiaexcelsa), pistachio nuts (Pistaciavera), macadamia nuts and Queensland nuts (Macadamia ternifolia), and products thereof, except: (a) nuts used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages.
9.	Celery and products thereof.
10.	Mustard and products thereof.
11.	Sesame seeds and products thereof.
12.	Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/litre expressed as SO2.
13.	Lupin and products thereof.
14.	Molluscs and products thereof.

(Source: Official Journal Directive 2003/89/EC)

3.2 Legal situation for wine labelling

New scientific studies conducted by the wine sector on allergenicity of milk and egg, used as fining agents in winemaking have been carried out lately. In June and July 2010 the International Organisation of Vine and Wine (OIV) made a request for labelling exemption regarding casein and ovalbumin used in the manufacturing of wine such as in clarification processing aids. Furthermore the European Commission submitted in

July 2010 to EFSA request for scientific opinions on the above mentioned substances (EU Directives, 1266/2010 and EFSA, 2004).
Taking into consideration that alteration in labelling rules influence industry, Directive 2005/26/EC and 2007/68/EC allowed the marketing of foods before May 31, 2009. This date was later extended until December 31, 2010. This period helps mainly small and medium-sized enterprises, which need an adjustment period to smooth the transition towards new labelling requirements. Furthermore this amending Regulation 2010/1266/EC permits scientists to further investigate the risks of those ingredients (EU Directives, 2010). This provisional temporary derogation is generally subject to the completion of scientific research to proof if allergen remains or not in the final product, before being confirmed as a permanent exemption.

On March 17, 2012 the OIV proposed the previous established detection limit of 0.50 mg/L to be reduced to 0.25 mg/L or 0.25 ppm (Resolution Comex 502, 2012 and Webber-Witt and Christmann, 2012).
Since July 1st, 2012 the European Commission accepted the proposed limit by OIV resolution through implementing Regulation 579/2012. Therefore all wines containing fining residues from ovalbumin, lysozyme or casein should be declared on the label if the quantity found in it is over or equals 0.25 mg/L (Christmann *et al.,* 2012). The official number by OIV for quantification is over or equal 0.50 mg/L (Regulation Comex 502, 2012).
There are consistent terms of declaring defined by the EU in each language of the community, thus pictograms may be applied (EU-REGULATION No 579/2012), to be partly seen on annexes chapter. The pictorial logos might also be used in combination with written declaration. The logos may be used in colour, grey or black and white.

Figure 1 Wine Allergens pictograms

Pictograms referred to in Article 51(2)

(Source: EU-REGULATION No 579/2012)

The same process happened with fish isinglass, which after scientific studies was exempt of declaration, also to be seen in Table 1 No.4 (b). It has been scientifically

established that isinglass is not likely, under specific circumstances, to trigger adverse reactions and therefore excluded from the labelling requirement (EU Directives, 2007).
At the present moment only sulphur dioxide (SO_2) has to be declared worldwide on all wine labels due to its allergic reaction potential. It has been mandatory for many years to identify this preservative due to its potential to cause adverse reactions in sulphite-sensitive asthmatic persons (Vally and Thompson, 2001).
Some other substances are already in force of declaration in some countries but not in all. For example in Australia and New Zeeland there are new allergen-labelling requirements in the *Australia New Zealand Food Standards Code* (the Code) for all food including wine (FSCANZ, 2011). This may be found on annexes chapter. The following legal additives and processing aids are often used for wine and wine products and are listed as allergenic substances under the Food Standards Code (GWL, 2011):

- added sulphites (such as SO_2/PMS) in concentrations of 10 mg/kg or more (preservative)
- casein and potassium caseinate (fining agent)
- egg white (fining agent) (including Lysozyme)
- milk and evaporated milk (fining agent)
- nuts (such as non-grape derived tannin that may be made from chestnuts).

The Code was amended on 28 May 2009 exempting isinglass (fish) for wine and beer. Accordingly, winemakers will no longer be required to declare isinglass on wine labels, as it is in Europe (FSCANZ, 2011).
In the USA it is not mandatory at the present moment to declare allergens on the label apart from sulphites. The American labelling laws are enforced by the Alcohol and Tobacco Tax and Trade Bureau (TTB, 2011).

3.3 Consumer protection

“People have the right to expect the food they eat to be safe and suitable for consumption. Foodborne illness and foodborne injury are at best unpleasant; at worst, they can be fatal” (*Codex Alimentarius*, 1997 p. 3).
This phrase above is written by FAO - Food and Agriculture Organisation and The United Nations World Health Organisation (WHO), together they claim this on their Codex Alimentarius; their main aim is to protect consumers.
Consumer protection is a role of governments and industry to encourage the implementation of general principals such as to ensure that consumers have easily-understood and clear information. This should be insured by way of labelling and other appropriate means, enabling to protect food from contamination and grow of foodborne pathogens by storing, handling and preparing it correctly. Since there is no

regulation for cross contamination the manufacturer has to prove its assiduousness. To protect their companies and consumers, big companies make use of diverse safety management quality systems such an ISO 22000 or IFS, BRC and QS from GFSI. All these systems may help to avoid or if necessary to trace allergen contamination (*Codex Alimentarius,* 1997).

3.3.1 HACCP

For the industry to protect the consumers it is important to know the laws, as the ingredients to be declared and the food safety as described in Hazard Analysis and Critical Control (HACCP). This General Principle document is recognized internationally as essential to ensure the safety and suitability of food for consumption (*Codex Alimentarius,* 1997).

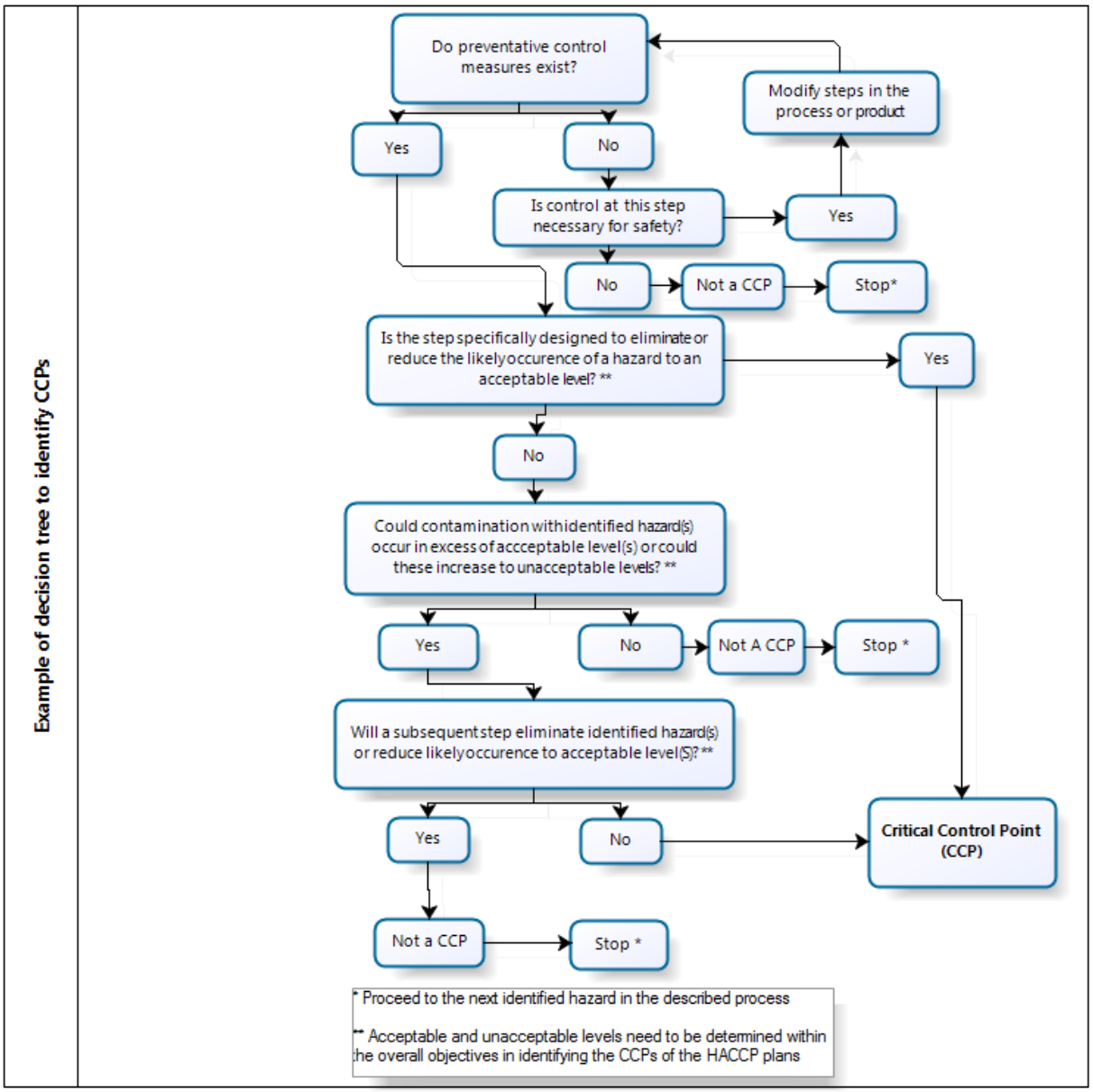
A HACCP is a system which identifies, evaluates and controls hazards which are significant for food safety. HACCP is science based and systematic. A hazard is a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect. Food hygiene is understood by all conditions and measures necessary to ensure the safety and suitability of food at all stages of the food chain, including individual primary producers, manufactures, processors, food service operators and retailers (*Codex Alimentarius,* 1997).

Principles of the HACCP system after *Codex Alimentarius*

- **Principle 1** - Conduct a hazard analysis
- **Principle 2** - Determine the Critical Points (CCPs)
- **Principle 3** - Establish critical limit(s)
- **Principle 4** - Establish a system to monitor control CCP
- **Principle 5** - Establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control
- **Principle 6** - Establish procedures for verification to confirm that the HACCP system is working effectively.
- **Principle 7** - Establish documentation concerning all procedures and records appropriate to these principles and their application

For the above-mentioned “Principle 4” of HACCP, it is possible to use an example of the decision tree as shown in following diagram.

Diagram 1 Example of decision tree to CCPs identification



(Source: Codex Alimentarius 1997)

The initial step in developing an Allergen Control Plan is to identify key leaders in the organisation who not only comprehend how ingredients flow through the facility, but also understand the very important significance of managing and controlling these ingredients at every stage from choosing suppliers to handling, storage, processing, packaging and labelling on a regular base (Food Allergy Research, 2008).

The OIV has a HACCP model for viticulture that has been approved as an EU Resolution in June, 2012. This Resolution considers the commercial requirements for products

with low food risk and the heightened concerns of the member states relative to the harmlessness of the products offered to the consumer (Resolution viti, 2012).

3.3.2 Residue and contamination

In wine there are two possible sources of allergens arising from animal proteins; residues coming from fining agents or from a possible contamination.
In general residues have various types of sources, yet in this work the main focus lies on residues that have their source from fining agents used throughout the wine making process. In other words residues are the remains of fining agents after chemical and physical operations.
A residue is different from a contaminant which is “any biological or chemical agent, foreign matter, or other substances not intentionally added which may compromise food safety or suitability” (*Codex Alimentarius*, 1997 p. 6).
Contamination, which is the injection or occurrence of a contaminant in food or food environment, could happen through cross contact during the production in which the final product will contain the contaminant, generally in traces. It can occur during transport or storage of raw material; insufficient cleanness of production equipment or even through dust coming from nearby or adjoining product lines. Contamination is in the most part of cases responsible for the phrase: “may contain traces of” declared on labels, as a matter of consumer and also producer protection, since producers have law consequences if any trace of allergen is found in its products and it is not declared (Busch *et al.* 2011). Cross-contact of foods with allergens has been shown to lead to allergic reactions in consumers on numerous occasions (Gern *et al.,* 1991; Jones *et al.* 1992; Yunginger *et al.,* 1983).

3.3.3 Threshold limit

The term “threshold” is defined differently in various fields of science. In toxicology for example it is the dose at, or below which, an adverse effect is not seen in an experimental setting, as for in methodology it is the limit of detection of an analytical method. In the field of statutory it is the establishment of a limit by statute, below which no regulatory action will be taken. Finally by dictionary definition it describes the intensity below which a mental or physical stimulus cannot be perceived and can produce no response (FDA, 2006).
Until the present moment there were only a few quantitative threshold values in the EU for food allergens on labelling apart from gluten and sulphur dioxide. Labelling for allergen traces is internationally well known and not always equal in all countries. In Switzerland, for example there is a threshold limit of one gram allergen element per kilogram, meaning that if this value is surpassed it must be declared. It is indifferent if

this element is coming from an ingredient used in the manufacturing process or from a cross-contamination. Meaning that even if a product passes through good manufacturing practices (GMP), but has become somehow an input of any allergen it might also have its declaration on the label - if over one gram per kilogram. Nevertheless these threshold limits are criticized by allergologists that want to protect very sensitive allergic subjects, because it is considered by them to be an eminent value (Busch *et al.* 2011).
Methods on how to diagnose clinical reactions will be explained later on in chapter 2.4.2.2. Even if it is established that an individual has an allergy to a food by skin test or by positive IgE serum, none of these diagnoses can reliably predict the level of patient sensitivity to low doses of the food. At the present, the level of individual sensitivity can only be determined using food challenge studies, detailed explained on chapter 2.4.2.2 in this work (FDA, 2006).
An example of well-defined thresholds is gluten, which is known as an intolerance trigger for people who have gluten sensitivity. After Codex-Alimentarius-Standards from 2008, there is a limited and defined threshold; every product containing an amount smaller than 20 ppm is considered to be gluten free, the amount from 21-100 ppm is defined as “reduced gluten” (Codex Standard, 2008).

3.3.4 VITAL-Concept

In Oceania there is an association called Allergen Bureau which works for the food industry in Australia and New Zealand. This organisation has developed a concept called Vital: Voluntary Incidental Trace Allergen Labelling, all information can be found under their website www.allergenbureau.net/vital. This concept shows a different approach for labelling of allergen particles present in correlated food. They have set different threshold limits for every allergen separately and according to three limits which may define exactly what to communicate on the label. The three limits are: under the minimum determined threshold limit, between both threshold limits and over the highest threshold level (VITAL-Concept, 2011). The following will show the classification of these three steps in specific levels:

- **Action level 1**: for allergen amount under the smallest limit, where no allergen has to be declared on the label
- **Action level2:** for allergen amount between the smallest and uppermost limit, here the label has a declaration of traces of the specific allergen, such as ‘contain traces of …’
- **Action level 3:** for allergen amount over the utmost limit, where the allergen is declared on the label as an ingredient.

The intention of this concept model is to keep unnecessary worries away from the consumers (VITAL-Concept, 2011).

In Europe there is a project based on the same principles of the Vital-Concept as used in Australia and New Zealand. It is called EU-VITAL. This European proposal project is defined by them as an improvement of the original concept in order to accomplish all requirements of European legislation. The model records contain the threshold levels as previously standardized in Europe DIN EN 15634-1 (EU-VITAL, 2011).

“EU-VITAL is a concerted initiative to improve and harmonize the declaration of food allergens. It is supported by food producers, distributers, analysing laboratories, governmental authorities and allergic consumers” (EU-VITAL, 2011).

EU-VITAL, which is based and consistent with European food allergen legislation, has been developed to identify coordinated “action levels” (Table 2) based on clinical thresholds for the labelling of allergens. They include allergens according to European guidelines (2007/68/EC and 200/13/EC), but they list fewer and diverse allergens as subject matter to labelling than the EU legislation (EU-VITAL, 2011). Action levels for the EU-VITAL are based on: clinical thresholds from DBPCFC challenges or on lowest LOAELs (Lowest Observed Adverse Effect Levels). These levels are related to total protein from the allergic food component testimonial. These clinical trials are the source for estimation of action levels. Furthermore with an addition of a 10 fold safety factor that is used to cover intra-individual discrepancies within the human population.

Table 2 Action levels table of EU-VITAL

the values for certain food allergens

allergen	action level 1	action level 2	action level 3
milk	< 50	50 - 500	> 500
lactose[1]	< 100	100 - 1.000	> 1.000
egg	< 20	20 - 200	> 200
soy	< 25	25 - 250	> 250
fish	< 100	100 - 1.000	> 1.000
peanut	< 8	8 - 80	> 80
tree nuts[2]	< 10	10 - 100	> 100
sesame	<10	10 - 100	> 100
crustacean	< 10	10 - 100	> 100
gluten [3]	< 20	20 - 100	> 100
celery	< 20	20 - 200	> 200
lupin	< 20	20 - 200	> 200
molluscs	< 20	20 - 200	> 200
mustard	< 20	20 - 200	> 200
SO_2	< 10	10 - 100	> 100

the values
the table shows values for the action levels of certain food allergens

the unit / dimension
mg allergenic substance / kg food [ppm]

[1] see position of association of german analytical chemists to declaration of lactose

[2] tree nuts according to Comission regulation (EC) No 68/2007
Hazelnut, Walnut, Brazil nut, Pecan nut, Cashew nut, Macadamia nut, Pistachio and Almond

[3] according Comission regulation (EC) No 41/2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten, according Codex Alimentarius

(Source: EU-VITAL, 2011)

Busch *et al.* defend the idea that allergen threshold adoption for foodstuff and furthermore no declaration requisite for allergens under a certain determined threshold, may be positive for allergic consumers. It would be a less restricted choice of food for sensitive consumers through preventive warnings and so it would be better for the industry and legal certainty due to increased monitoring.

A contemporary study released in June 2013 and published in 2014 points the eliciting dose for an allergic reaction of 1% of the population was 0.1 mg for cow's milk and 0.03 mg for egg (Allen, K. *et al.,* 2014).
The study goals were to launch orientation doses for 11 commonly allergenic foods to guide a coherent approach by manufacturers based on all publically available valid oral food challenge data. The methods used for the study were established from statistical dose-distribution modelling of individual thresholds of patients in a dataset of more than 55 studies of clinical oral food challenges to permit valuation of the

representativeness of the data used. Included in this study were milk and egg which are of extreme importance to this work (Allen, K. *et al.,* 2014).
"These reference doses will form the basis of the revised Voluntary Incidental Trace Allergen Labelling (VITAL) 2.0 thresholds now recommended in Australia." (Allen, K. *et al.,* 2014). Finally these new levels will permit producers to put on reliable protective labelling and offer improved consumer assurance in their validity and reliability, as well as improving consumer well-being (Allen, K. *et al.,* 2014).

3.4 Food Reactions

Food illness is not something new; it is known since ancient times and has been already mentioned by Hippocrates (Chabot, 1951, in Høst, 1997). The term "allergy" itself was first mentioned by Clemens von Pirquet in 1906, from the ancient Greek words *ἄλλος*: allos meaning "other/different/changed" and *ἔργον*: ergon meaning "reaction/work" (Pirquet, 1906 in Lilja and Wilkmann, 1998).
First of all it is important to mention that even between allergy experts there are constant discussions on appropriate nomenclature and terminology used to characterize allergic and allergy like reactions, which may be sometimes confusing, as claimed by Johansson. He points out that without a universal understanding and a strict use of language to classify allergic disease, neither science nor patient care can be optimal. The World Allergy Organization (WAO) has worked together with a review committee with the final goal of improving communication in the field of allergy. The European Academy of Allergy and Clinical Immunology (EAACI) and the Nomenclature Position Statement (NPS) created a working group with appointed mission to standardize the nomenclature of allergy. This revision is expected to be used not only by physicians, in learning or research but also by patients and persons involved in allergy (Johansson *et al.,* 2004).
While adverse reactions may occur for a range of toxicological, immunological or metabolic reasons only a small portion of these are related to real food allergies. Therefore consumers may wrongly believe that a large range of adverse reactions are linked with the intake of foods to be "food allergies" (FDA, 2006).
The above proposed the revision of nomenclature as shown in Table 3. This nomenclature is based on the mechanism initiating a reaction.

Table 3 Allergy revised nomenclature for global use

General Terms	
Hypersensitivity	describes *objective reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons. Can also be called sensitivity.*
Allergy	*a hypersensitivity reaction initiated by specific immunologic mechanisms.* When other mechanisms can be proven the term *nonallergic hypersensitivity* should be used
Atopy	*a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce Immunoglobulin E (IgE) antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema*
Allergen	*an antigen causing allergic disease.*
Allergic diseases	
Asthma	if resulting from immunological reactions should be called *IgE allergic asthma*
Rhinitis	if resulting from immunological reactions *IgE allergic rhinitis*
Conjunctivitis	*allergic rhinoconjunctivitis*
Dermatitis	*atopic eczema* or *atopic dematitis*
Urticaria	if resulting from immunological reactions should be called *IgE allergic asthma*
Food hypersensitivity	*food allergy, IgE-mediated food allergy* and *nonallergic food hypersensitivity* if not IgE mediated.
Anaphylaxis	*a severe, life-threatening generalized or systemic hypersensitivity reaction.* If resulting from immunological reactions should be called *allergic anaphylaxis*

(Source: Table based on Johansson, 2004)

3.4.1 Nonallergic food hypersensitivity (food intolerance)

Hypersensitivity reactions that are related to food but do not present IgE mediators can also be referred to as nonallergic food sensitivity (Johansson *et al.,* 2004). It consists of any atypical reaction resulting from the ingestion of a food where reactions are not factual food allergies. Those reactions are caused, for example by the occurrence of toxic compounds such as histamine in seafood or from metabolic disorders, such as lactose or gluten intolerance. Gluten contains gliadin and glutelin proteins that are found in all forms of wheat. Similar proteins are found in rye, barley and oats. Further well known foods that may cause intolerance are: other gluten containing grains, cow's milk as for other dairy products and corn foods. Usually, reactions not involving immune response are named food intolerances and there are a

larger number of individuals suffering from these nonallergic reactions than individuals with true immunological reactions (Johansson *et al.,* 2004; FDA, 2006 and Sampson, 2004).

Food intolerance can also occur when the body lacks the enzyme necessary to digest a specific food or has a genetic disorder. There are specific reactions involving immunological responses, but they are likewise non-IgE mediated hypersensitivity such as celiac disease and food protein-enterocolitis (Sampson, 2004 and FDA, 2006).

Celiac disease

Celiac disease (CD) or celiac sprue is known as permanent gluten intolerance; it is a genetic autoimmune disorder that causes an inflammatory disease of the upper small intestine, more specific to the absorption tissue, also called villi (Walker-Smith, 1980). Injured villi do not effectively absorb essential nutrients – carbohydrates, proteins, fats, vitamins, minerals and, in some cases, bile salts and water. If CD is left untreated, injure to the small bowel can be persistent and life threatening, causing an augmented risk of linked disorders – both nutritional and immune related (Celiac, 2010). Celiac Disease can appear at any time in a person's life. Infants and young children with CD may often exhibit growth breakdown, vomiting, bloated abdomen, behavioural changes and failure to thrive. Characteristic symptoms may be abdominal cramping, intestinal gas, distention and bloating of the stomach, chronic diarrhoea or constipation (or both), steatorrhoea – fatty stools, anaemia – unexplained, due to folic acid, B12 or iron deficiency (or all), unexplained weight loss with large appetite or weight gain (CDF, 2012).

Food protein induced enterocolitis syndrome

Food protein induced enterocolitis syndrome (FPIES) normally concerns infants and young children. It can be caused by food hypersensivity, and causes symptoms of vomiting and bloody diarrhoea that leads to dehydration and shock after the consumption of certain foods (More, 2011).

3.4.2 Food-induced allergic reactions (food allergy)

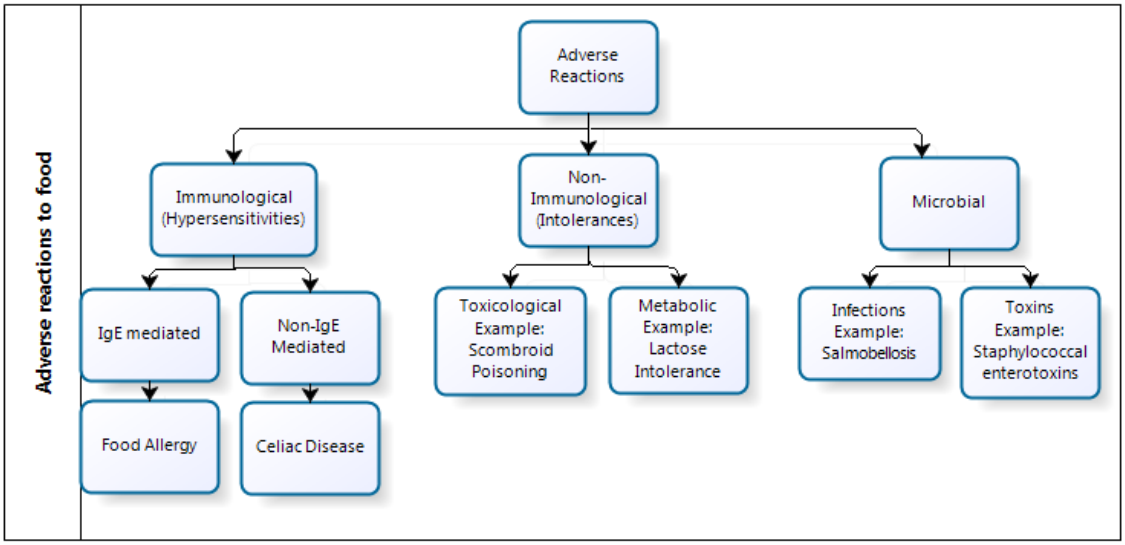
Food allergy is a hypersensitivity reaction initiated by specific immunologic mechanisms, IgE-mediated immune responses resulting from ingestion of specific foods, when the body's immune system reacts to otherwise harmless substances in certain foods. Allergy can be antibody-mediated or cell-mediated (Johansson *et al.*, 2004; FDA, 2006 and Sampson, 2004).

Food allergy continues a foremost cause of anaphylaxis treated in emergency departments in a number of nations, and the community has increased its observation of the problem (Sampson, 2004). The indicators and symptoms linked with these

reactions may vary from oral irritation and swelling to cardiovascular collapse (Jackson, 2003).

Young children are the mostly affected by food allergies and approximately 90% of these allergies are caused by eight foods: eggs, cow's milk, peanuts, soy, wheat, tree nuts, fish, and shellfish. With the exception of peanut allergy, the majority of children outgrow their food sensitivities (UMMC, 2012).

Diagram 2: Adverse reactions to food



(Source: FDA, 2006)

As mentioned above an allergic reaction originates from an uncharacteristic, or overstated, immune system answer to specific antigens by producing antibodies (Sampson, 1999). An allergen or antigen is a substance that reacts with the products of a specific immune response while an antibody is a specific protein which is produced in response to an immunogen and which reacts with an antigen. An immunogen is a substance that induces a specific immune response. It is also capable to stimulate or provoke an immune response or producing immunity. An antigen on the other hand is able to combine with the products of an immune response once they are made.

Allergic reactions are highly specific; this specificity is due to the epitope or antigenic determinant, which is that portion of an antigen that combines with the products of a specific immune response. In a reaction involving, for example milk protein in a susceptible person, these proteins would be the antigen, the body plasma B cells responsible to induce immune response producing IgE, would be the antibodies. IgE cells normally attach to mast cells. These cells contain granules of chemicals such as histamine.

To better explain the above-mentioned reaction it will be divided in two phases for this immune response:

1. Sensitisation
2. Elicitation

Sensitisation will occur as a response to an allergen (specific food). Elicitation of an allergic reaction follows on a subsequent contact to the identical allergen.

The antibody recognises a unique part of the foreign target, or the antigen. These mechanisms take place in susceptible subjects, their immune system plasma cells located in lymph nodes produces significant amount of allergen IgE antibody against a particular food protein. IgE cells attach themselves tightly to mast cells surface, also called basophiles, through the high-affinity IgE receptor identified as FcεRI. A second contact to the same allergen and further binding of antigen to IgE primed master cells will release chemical mediators (e.g., histamine and leukotrienes). The chemicals released may be responsible for the symptoms of the allergic reaction, because they cause inflammatory molecules. The concentration and type of allergen, and the way of exposure are responsible for the severity and consequences of reactions (FDA, 2006; Janeway, *et al*., 2001 and Taylor and Hefle, 2001). Following figures describe with drown an allergic reaction.

Figure 2 Sensitisation and elicitation of an allergic reaction

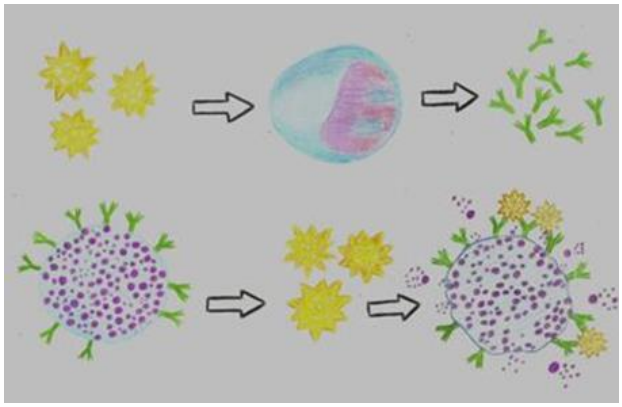
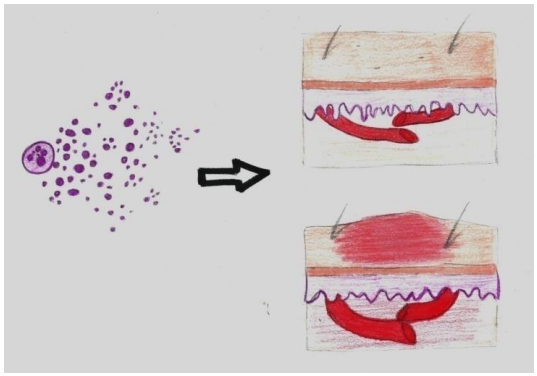


Figure 3 Symptoms of the allergic reaction on skin



(Source: Drawn by Juliana Mazarro Webber)

3.4.2.1 Symptoms

The clinical symptoms or signs of true food allergic reactions usually involve the skin and the gastrointestinal way - symptoms including urticaria, angiooedema, eczema, itching of lips, tongue or palate, diarrhoea, stomach upset, indigestion, bloating, gas, nausea, vomiting, and cramps. The reactions array from serene irritation to brutal life-threatening respiratory suffering and shock or as already mentioned from oral irritation and swelling to cardiovascular collapse. Typically it begins just after eating and normally not longer than two hours following ingestion of the particular food. The expression “anaphylaxis” is used to explain multisystem severe reactions to an allergen needing urgent medical intervention (Jackson, 2003).
Severity of an allergic reaction is affected by several causes that embrace genetic predisposition, age, type of food allergen, nature of any food processing environment, and physiological conditions (Sampson, 2003; Taylor and Hefle, 2001).

3.4.2.2 Diagnosis

There are different ways to diagnose allergy, basically three groups of tests that can be used as a diagnosis if linked to a history of clinical reaction to a food:

1. **Skin allergy test**
   Group subdivisions:
   - Skin prick test (SPT)
   - Skin patch test
   - Intradermal test
2. **Allergen-specific IgE test**
3. **Oral food challenge (OFC)**
   Group subdivisions:
   - Double-blind placebo-controlled food challenge (DBPCFC/DBPC)
   - Single-blind placebo-controlled (SBPCFC/SBPC)
   - Open Oral Food Challenge

Skin allergy test

This is one of the most common methods of allergy testing; the scratch test or skin prick test. The test involves placing a small quantity of the suspected allergy-causing matter (allergen) on the skin (usually the forearm, upper arm, or the back), and then scratching or pricking the skin so that the allergen is introduced under the skin surface with a sterile needle or a pin. The skin is observed closely for signs of a reaction, which typically embraces swelling and redness of the spot if the allergen skin test is positive. With this test, several suspected allergens can be tested at the same time, and results are usually obtained within about 20 minutes (NLM, 2012).

Intradermal injections are done by injecting a small amount of allergen just beneath the skin surface. After waiting ten minutes, the skin is examined for a reaction. This procedure is repeated using two to three dilutions of the extracts, getting subsequently stronger each time. Once the skin reacts positively to a particular allergen(s), the test is complete. In the skin patch test where the patch contains the allergen that is placed on the skin for up two days. Both tests are less common or less used for food allergy diagnosis (Medbroadcast, 2012).
A negative test does not directly mean that the subject is not allergic; it may be simply that either the right concentration was not used or the body failed to elicit a response.

Figure 4 Skin prick test or scratch test

(Source: NLM, 2012; MedBroadcast, 2012)

Allergen-specific IgE antibody test

This is a blood test also known as allergy screen or RAST test, meaning Radio Allergosorbent Test. In some other allergen diagnosis tests there is also the potential for severe reactions and in these cases, the allergen-specific IgE antibody test can be an alternative, as it is performed on a blood sample and does not have an effect on the person being tested.

Blood tests look for antibodies and the most common type test used is the ELISA. It measures the blood level of IgE that the body may make in response to certain allergens. Other lab testing methods, such as RAST or an immunoassay capture test (ImmunoCAP), may be used to provide more information (AHC, 2012).
Elevated results usually indicate an allergy, but even if the specific IgE test is positive, a person may or may not ever have an actual physical allergic reaction when exposed to that substance. The amount of specific IgE present does not necessarily predict the potential severity of a reaction. A person's clinical history and additional medically supervised allergy tests may be necessary to confirm an allergy diagnosis (AHC, 2012).
This test can also be used to monitor desensitization (immunotherapy) or to test if a person has outgrown an allergy. However, the level of IgE present does not correlate to the severity of an allergic reaction, and someone who has outgrown an allergy may have a positive IgE for many years afterwards.
Negative results indicate that a person probably does not have a "true allergy", namely an IgE-mediated response to that specific allergen. The results of allergen-specific IgE antibody tests must always be interpreted and used with prudence, because even if an IgE test is negative, there is still a small chance that a person does have an allergy (AHC, 2012).

Oral Food Challenge studies

The DBPC is considered to be best standard diagnostic assess for establishing clinical reactivity to low concentrations of an allergen. This test is named “gold standard” of food allergy diagnosis. This challenge is called “double-blind” because neither the patient nor the physician/researcher knows which test foods contain the allergen. In SBPC only the physician knows which food contains the allergen and in the open challenge, both the subject and the physician know which food test contain the allergen. The advantage of DBPC is that bias may not play a role in interpreting patient reactions, because patients do not know what they are getting, their belief about what will happen does not change the results (FDA, 2006).
Even though currently the level of individual sensitivity can only be determined using food challenge studies; DBPC, SBPC or open challenge, most oral challenge studies are intended to launch a diagnosis of food allergy rather than to establish safety (Taylor et al. 2004; FDA, 2006). Test participants can pass or fail the challenge. Only if they are able to tolerate 100% of the intended dose without untoward effects they pass, while patients that show signs of clinical reactivity fail the test. The identification of patients with clinically reactive food allergies remains difficult for the allergist, because food challenges can be traumatic for the patient, are time-consuming, and are potentially dangerous. Apart from the risk, challenges performed under guidance of an

experienced practitioner and in a properly equipped setting are considered to be reasonable (Perry *et al*., 2004).

Perry *et al.* have undertaken 584 challenges in 382 patients resulting on 253 (43%) failed challenges. None of the patients required hospitalisation and all symptoms were reversible. Research was based on four diverse food allergens. In the same study researchers found no relationship between reaction severity during failed food challenges and food-specific IgE levels, as already mentioned above. Even though food-specific IgE levels serve as useful forecasters of challenge result, researchers reinforce the fact that clinicians should be aware that failed challenges can happen at any level of IgE, together with below detection, concluding that IgE level may have slight or no value on prediction reaction severity.

For ethical reasons patients with very high food allergen IgE serum titres are often excluded from challenge studies, so do individuals with a history of anaphylaxis to food (Taylor *et al.*, 2002). At the same time this may be critical while trying to estimate adverse-effect level, because it could be argued that these patients may be among the very most sensitive and for study matter the representativeness might be inappropriate (Taylor *et al.*, 2002).

DBPC Studies

(Factors affecting the determination of threshold doses for allergen foods)

Allergists know that contact to small quantities of offending food can bring out adverse reactions. The threshold dose for provocation of such reactions is often considered to be nil. Nevertheless, due to some limitations on manufacture, food may sporadically contain trace residues off allergen. As it is not simple to predict if these tiny quantities are dangerous, Taylor *et al.,* (2002) studied the quality and quantity of some existing clinical data. The study was based on doses for commonly allergic foods and summarise the consensus to be reached on establishment of threshold doses for specific foods. He found out considerable data in clinical files relating the threshold doses for peanut, cow's milk and eggs. But he concludes that because these data were often obtained by means of different protocols, the estimation of a threshold dose was very difficult.

The study done by Taylor *et al.* (2002) also highlighted the fact that the farm-to-table food production, processing and distribution chain needs improved information on the threshold dose for provocation of allergic reactions to food. The proliferation of precaution labels (e.g., "may contain") is an industry response to the existing uncertainty. But with no information on threshold doses, it is hard for the food industry to perform hazard assessments and to centre their quality control efforts where the advantage to the allergic consumer would be greatest. Study also claims that in case of food contaminated with trace residues of an allergic food, such histories

must be connected with a quantitative analysis of the amount of the allergic food found in the implicated product. But there is few approximations done involving rather a small number of patients. The study suggests that this probably occurs because of the lack of simple methods for the analysis of the implicated food product for residues of commonly allergenic foods.

Controlled clinical challenge trials can obtain the finest approximations of the threshold dose for various allergenic foods. Only few studies were found with the purpose to determine the threshold dose and as already mentioned in this work commonly challenges have been conducted on diagnosis purposes, rather than for determining the lowest provoking dose. Nonetheless, typical protocols engross starting at doses that are one half or less the amount of the offending food estimated by the patient to incite symptoms (Taylor *et al*., 2002).

Based on clinical groups Taylor *et al.* (2002), used information from DBPCFCs used for diagnostic purposes and he found valuable data on cows' milk and egg among others.

Table 4 Results of accumulated data on the lowest provoking doses in OFC

Food	Patients No.	Test	Lowes provoking dose (LPD)	No. of patients reacting to LPD	Range of LPD for these patients
Egg	281	DBPC and SBPC	1 mL of liquid whole egg	2	1 mg to 5 g
Milk	299	DBPC,SBPC and open	0.02 mL (different forms of milk)	21	0.02 mL to over than 100 mL

(Source: Taylor *et al.* 2002)

The study conclude that it is clearly possible to define that the threshold dose for allergens is finite, measurable and above zero. Studied data might be premature to define a threshold since they used diverse protocols and were diagnosis oriented. Furthermore, Taylor *et al.* (2002) gives as major reason for not estimating a threshold dose; the basis of the on hand data is that the no-observed adverse-effect level (NOAEL) was not established for the vast majority of patients. In risk assessments used by WHO, FAO among other worldwide regulatory agencies to establish acceptable daily intake, also called virtually safe doses, for food additives, the NOAEL is first established on the basis of experimental research with animals or humans. Because the DBPFCs were being performed for diagnostic purposes, only the LOAEL was recorded. For risk purposes of estimating a threshold dose, it would be preferable to have information on the NOAEL. The highest dose in the DBPCF that did not elicit an

adverse reaction must be known to determine the NOAEL. The most susceptible patients of these studies concerned in challenges reacted to the first lowest dose used. This dose is thus the LOAEL, and uncertainty exists about how much less of the offending food would be necessary to reach the NOAEL.
In sum, the study done by Taylor *et al.* (2002) conclude that threshold for peanut, egg and cow's milk appear to be in the low milligram range or higher for most individuals with allergies to those foods. Thus these individuals can ingest food without any untoward reactions. As a concluding note the study recommended that worldwide efforts should be undertaken to establish threshold doses for commonly allergic foods using standardized clinical challenge protocols and using a wide range of affected patients as possible.

3.4.2.3 Frequency of allergy

Existing figures show that probably <2% of the adult population suffers from food allergy (Niestijl *et al.*, 1994 and Young *et al.* 1994).
Some studies show that children are likely to outgrow allergies in adulthood (FDA, 2006).

Table 5 Allergy Prevalence in USA

Age Group	Percentage of the population		
	all allergens	milk	Egg
Children	6.0	2.5	0.8
Adult	3.7	0.3	0.6

(Source: FDA, 2006)

Accurate numbers of victims are difficult to determine but projections can help to envisage the dimension of the problem. Yocum *et al.* (1999) showed in a four-year time-period population base study in the USA, an anaphylaxis occurrence rate of 30 of 100 000 persons per year was reported. From this same data, the number of 29 000 anaphylactic episodes because of food in the USA per year could be extrapolated, with approximately 150 expected deaths per year (Bock et al. 2001). Reported to the European population this number would be above 200 deaths per year. A number of over 20 million of the European population are estimated to suffer from food allergy (Eigenmann, 2003).

3.4.2.4 Treatment

There is no treatment for allergies apart from avoidance of the allergen. The management of food allergies continues to consist of educating patients on how to keep away from relevant allergens, to be familiar with early symptoms of an allergic

reaction in case of an accidental ingestion, and to initiate the appropriate emergency response (Sampson, 2004).

3.5 Allergens in wine

Wine is the product obtained exclusively from the total or partial alcoholic fermentation of fresh grapes, whether or not crushed, or of grape must.

There are only few individual case studies reported from immunologically mediated reactions after consumption of wine. Nevertheless there is a recent study by Wigand *et al.* (2012) that studies the frequency of wine intolerance of the adult population in Mainz, Germany. This was a cross-sectional study based on a questionnaire survey, with 4,000 randomly selected individuals. Asked questions were related around alcohol consumption and the incidence of various intolerance and allergy-like symptoms after the consumption of wine. In their results they found out that 948 persons aged from 20 to 69 years (27.7%) were comprised in the analysis. 68 (7.2%) of the 948 study takers reported an intolerance to wine and/or allergy-like symptoms after wine consumption. A wine intolerance was specified more often by women (8.9%) than men (5.2%) ($p = 0.026$).

People who shared a wine intolerance are more likely to report other intolerances especially against beer and alcohol in general. Allergy-like symptoms were observed more frequently after the enjoyment of red wine. Redness and itching of the skin as well as a stuffy nose were the most frequently mentioned reactions (Wigand *et al*., 2012).

The prevalence of wine intolerance was higher than expected. From the collected parameters, it suggests less of an immunological mediated allergy, but rather intolerance to alcohol, biogenic amines or other ingredients (Wigand *et al*., 2012).

3.5.1 Grape origin

The majority of the wine proteins originate from the grape, while some are released from yeast or bacteria. Grape proteins found in wine are mainly pathogenesis-related proteins (PRs). These are formed by plants as a resistance against fungal pathogens and are produced because of their potential as bio-control agents. Their origin is through accumulation in the grape berry during ripening and is expressed as a result of biotic or abiotic stress (Waters *et al.*, 1996 and Palmisano *et al.*, 2010).

Waters et al. (1996) showed by SDA-PAGE the presence of proteins in wine demonstrating homology to thaumatin like proteins and chitinases, at least in two forms. This last enzyme-protein has antifungal properties against chitin, a major structural component of many fungal cell walls (Boller, 1987 in Waters, 1996). These proteins are highly resistant to proteolytic attack and low pH of wine, for these reasons

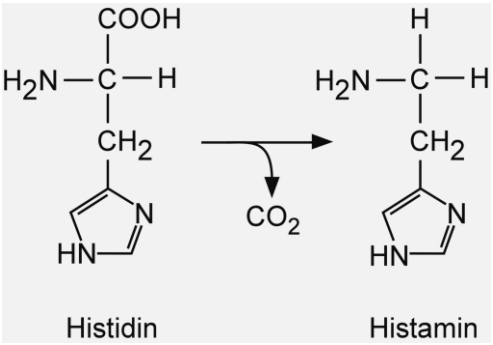
they are not lost during vinification and remain in wine and they are discussed as potential grape and wine allergens (Feuillat and Ferrari, 1982 in Waters, 1996).
Recent studies done by Palmisano *et al*. (2010) suggested that above mentioned proteins are coated with sugars (glycoproteins) and bioinformatic of the study showed that structures identified in grape glycoproteins are similar to known plant allergens, which could lead to potential allergic cross-reactivity in wine.
Pastorello *et al.* (2003) point out in a study that grape and wine might cause allergic reactions in sensitive patients and that the major allergen from the grape is endochitinase, which is also in wine.
Reported cases of allergic reactions to grapes are negligible and studies on the relationship between wine and allergic reactions are rare just as rare. Grape allergy is often cross-reactivity with other fruits proteins, especially observed from *Rosaceae* family, for which the lipid transfer protein (LTP) is responsible (Wigand, 2008). Allergy to Prunoideae (*Rosacea* family) fruits; peach, apricot, plum and cherry, is one of the most common food allergies in southern Europe. All these fruits cross-react in vivo and in vitro, as they divide their major allergen, a 9 kD lipid transfer protein. LTP is a true pan allergen with a degree of cross-reactivity. Due to its extreme resistance to pepsin digestion, LTP is a potentially severe food allergen. LTP is an officially acknowledged allergen in grapes and an LTP cross-reacting with the peach major allergen. This protein could be identified in some red wines, whereas its presence in white wine is rare (Pastorello, 2001 and 2003; Asero, 2001 and Wigand, 2011).

3.5.2 Microorganism origin

3.5.2.1 Biogenic-amine

Biogenic amines are low molecular organic nitrogenous bases that contain biological activity; they have aliphatic or cyclic structures. They are originated from amino acids decarboxylation through substrate-specific decarboxylase enzymes. They receive this name because they are formed or degraded during the normal metabolism of living organisms; animals, plants and microorganisms. Although biogenic amines are needed for many critical functions – allergic response, neurotransition and vascular permeability in man and animals, consumption of food containing high amounts of these amines can have toxicological effects. Allergic-like reactions have been reported with respect to wine and biogenic amines (Wigand *et al*., 2012).

Figure 5 Histidine decarboxylation



(Source: http://www.orthomedis.ch/F2.gif)

The histamine intolerance is not IgE mediated, which is confirmed by the lack of IgE antibody and Prick tests (Wantke, 1993). The symptoms of histamine intolerance are allergy-like symptoms. In patients in whom these symptoms are developed, there was an increase of histamine in plasma after consumption of red wine compared to asymptomatic control subjects detected (Wantke, 1994). In patients with histamine intolerance there might be the existence of reduced histamine degradation, caused by a deficiency of diamine oxidase, the main enzyme for the metabolism of histamine in the gut (Bieganski *et al*., 1983).

Biogenic amines play an important role in physiological functions but they are possible threats of toxicity to humans and consequent trade repercussions. They may exert vasoactive effects, psychoactive effects or both. Although there are differences in individual susceptibility to intoxication by biogenic amines, symptoms that can occur after excessive oral intake of biogenic amines comprise nausea and vomiting, headaches, discomfort, hot flushes, cold sweat, heart palpitations, red rash, high or low blood pressure; hyper-and hypotension (Rice *et al*., 1976 in Brink *et al.*, 1990; Smit, 2008 and Smit *et al.*, 2008).

Wine intolerance may not be solely correlated to histamine content. Kanny *et al*., 2001 showed in a clinic study with oral provocation that there is no relation between wine intolerance and the amount of histamine found in wine. DBPC tests were done, with oral provocation in that the amount of histamine found in wine was reached till 13.8 mg/L.

There are studies showing the presence of biogenic amine in wine already in 1965, in Germany. The reason why biogenic amines received more attention in alcoholic drinks is because alcohol and acetaldehyde have been found to increase its sensitivity in humans since they can directly or indirectly inhibit the enzymes responsible for the detoxification of those compounds. (Marquardt *et al.*, 1965; Suárez, 2004 and Moreno-Arribas *et al.*, 2009).

In wines the main biogenic amines are showed in Table 6:

Table 6 Biogenic amines found in wine

Biogenic amine	IUPAC name	Amino acid
Histamine*	2-(1H-imidazol-4-yl)ethanamine	Histidine
Tyramine*	4-(2-aminoethyl)phenol	Tyrosine
Putrescine*	butane-1,4-diamine	Ornithine/arginine
Ethanolamine	2-Aminoethanol	Serine
Phenethylamine	phenylethan-2-amine	Phenylalanine
Cadaverin	1,5-diaminopentane	Lysine

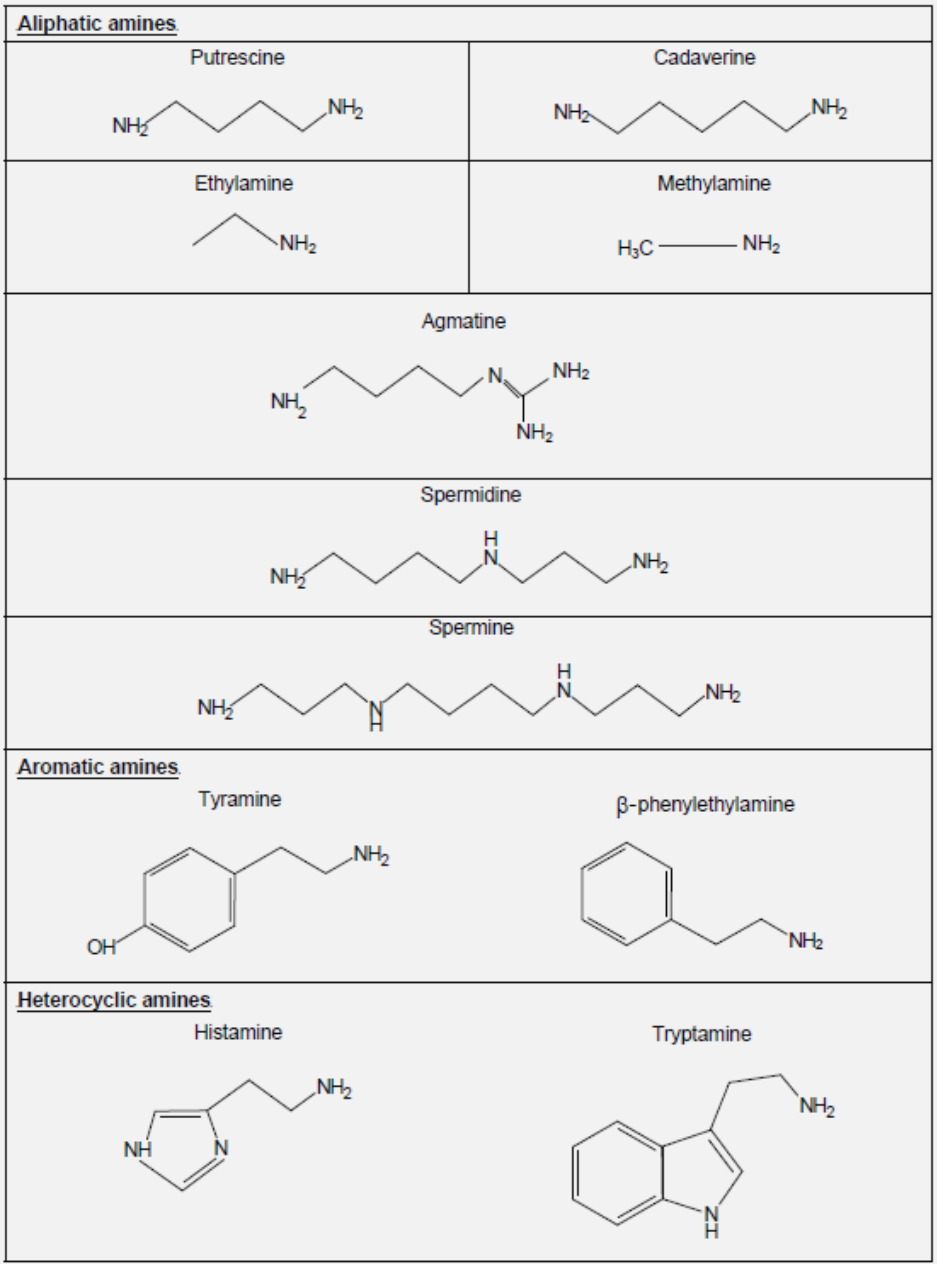
(Source: Suárez, 2004 and Ribéreau-Gayon, 2006) *Most common in wine

Smit *et al.*, 2008, claims that apart from those biogenic amines showed on Table 6 spermidine, spermine, agmatine and tryptamine can be associated with wine (Figure 6).

Usually, the toxic dose in alcoholic drinks is considered to be between 8 and 20 mg/L for histamine and 25 and 40 mg/L for tyramine, but as little as 3 mg/L phenylethylamine can cause harmful physiological consequences (Moreno-Arribas *et al.*, 2009).

As biogenic amines are derived from aromatic or cationic amino acids they all have one or more positive charge and a hydrophobic skeleton (Brink *et al.*, 1990).

Figure 6 Chemical structures of oenological important biogenic amines



(Source: Smit *et al.,* 2008)

Biogenic amines are found in fermented food and beverage, such as cheese and beer. In wine they can be formed from their particular amino acid precursor by various microorganisms present in wine, at every phase of production, ageing or storage. The microorganisms responsible for its formation can be yeasts or acid lactic bacteria, through enzymatic activity. Red wines have commonly higher levels of biogenic amines than white wines (not biologically des-acidified), as it normally undergoes malolactic fermentation (Ribéreau-Gayon, 2006 and Smit, 2008).

Smit 2008 states that there are contradictory statements among authors regarding the production of biogenic amines by bacteria in wine. Some claim that *Oenococcus oeni* (lactic acid bacteria) dominant species that survive alcoholic fermentation and widely used as culture in the wine industry, are not responsible for biogenic amine in wine, while others claim the contrary. He concludes that the ability of lactic bacteria to produce amine appears to be strain-dependent, and not a species precise attribute. Finally he suggests that decarboxylase activities are randomly distributed within the different species of *Lactobacillus, Leuconostoc, Pediococcus* and *Oenococcus*.

Inoculation with *O.oeni* starter cultures that are incapable of producing biogenic amines might be a workable option for the control of these compounds in wine (Martín-Álvarez *et al.* 2006).

Concerning yeast on the production of biogenic amines, studies are just as contradictory as for acid lactic bacteria. Some authors say that yeasts are not related to biogenic amine production while others confirm it (Herbert *et al*, 2005; Marcobal *et al.*, 2006; Torrea *et al*., 2002 in Smit, 2008). Among those authors in which studies confirm the production of biogenic amine there is a theory that the production is strain dependent, as it can be seen on following table.

Table 7 Production of biogenic amine by different wine yeast species

Yeast species	Average total biogenic amines (mg/L)	
	Caruso *et al*. (2002)	Granchini *et al.* (2005)
Saccharomyces cerevisiae	12.14	13.7
Kloeckera apiculata	6.21	9.7
Candida stellata	7.73	7.8
Metschnikowia pulcherrima	9.6	13.3
Brettanomyces bruxellensis	15.01	20

(Source: Smit, 2008)

The degree of biogenic amines formed in wine mostly depend on the vast quantity of amino acid precursors in the medium, since on the whole, biogenic amines increase with a raise in amino acids. Amino acid content may be influenced by vinifications methods, grape variety, geographical region and vintage, furthermore extended permanence of wine with the lees influences the concentration of amino acids and favours the amine production (Moreno-Arribas *et al.*, 2009).

Although the legal limits have not yet been established for any biogenic amine, some countries have drawn up their own recommendations, especially for histamine. Austria and Switzerland (Regulation 2000-2732 955, from EDI) reject wines over the recommended limit (EDI, 2012; Moreno-Arribas *et al*, 2009).

Table 8 Discussed limit for biogenic amines in different countries

Country	Biogenic amines limit (mg/L)
Switzerland	10*
Austria	10*
Germany	2
Holland	3
Belgium	5 to 6
France	8
*tolerance value ('Toleranz wert') for wines	

(Source: Moreno-Arribas *et al.*, 2009)

3.5.2.2 Ethyl Carbamate and Ochratoxin A

Other secondary metabolites that could pose a health risk to humans include ethyl carbamate and ochratoxin A, a mycotoxin. Both are by-products that can be found in thresholds in wine. They are not directly related to allergy but they are known to be toxic and suspected carcinogen (Suárez-Lepe and Íñigo, 2004).

Ethyl carbamate, also known as urethane occurs naturally in fermented foods and beverages. In wine ethyl carbamate is produced from natural reaction between urea and ethanol. This reaction occurs much faster at higher temperatures. Urea is formed when wine yeast or bacteria metabolise arginine, present in grape juice. Arginine, along with proline, is generally the major amino acid found in grape juice. All identified yeasts possess the enzymatic capacity of arginase that catalyzes the division of arginine to ornithine and urea. Investigation using radioactivity labelled urea has monitored the formation of ethyl carbamate in wine. The source of urea was investigated during fermentation. Study concluded that urea is formed from arginine during vinification (Monteiro *et al.*, 1989).

There have been other reported ways of ethyl carbamate formation, such as through microbiocidal agent DEPC - diethypyrocarbonate, commercially called *Baycovin* or other natural ways by lactic acid bacteria, for example (Ought, 1975 in Uthurry *et al.,* 2004). Thresholds up to 6 ppb of ethyl carbamate found in wine are reported to be harmless (Sponholz *et al.,* 1991). According to Canadian legislation wine should not exceed 30 µg/L of ethyl carbamate. A study done by Uthurry *et al*. (2004) have shown that the maximum level of ethyl carbamate in some Spanish red wines were <25 µg/L.

Figure 7 Ethyl Carbamate formation

(Source: Wine and Vine, 2012)

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species, including *penicillium* and *aspergillus,* moulds that may contaminate agricultural property either before harvest or during storage. Its chemical structure consists of a chlorine-containing dihydroisocoumarin linked through the 7-carboxyl group to l-β-phenylalanine. It is carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly has neurotoxic properties. It has also been associated with kidney diseases of humans. It is a natural component of many herbal products and is in cereals, coffee beans, cocoa and found in dried fruit. Codex Alimentarius Commission estimates that 15% of the total intake of OTA is due to wine, on the basis of limited data (EU Directives, No. 1881, 2006 and Castellari, 2002).
Regulation(EC) No 1881/2006 of 19 December 2006 lays down the maximum levels for these substances in cereals and cereal products, dried vine fruit, roasted coffee, wine, grape juice, spices, licorice and baby food. The tolerable weekly intake (TWI) for ochratoxin A is 120 ng/kg body weight (EU Directives, No. 1881/2006).

3.5.3 From additive or fining agent origin

3.5.3.1 Sulphite (SO_2)

A group of sulphur-containing compounds known as sulphites are present in wine. There are no wines that are completely sulphite-free, because they are naturally occurring fermentation by products. Sulphites help to inhibit the growth of harmful bacteria, avoid oxidation and have other effects that are positive on wine conservation (Dietrich *et al.,* 2005). Complementary sulphites are as well added to wines as preservatives. In susceptible individuals, sulphites can trigger asthma attacks or even anaphylactic shock. At a concentration of just 10 ppm, the wine must legally carry the warning label; it is mandatory in almost all wine producing countries using the terms such or similar to "contains sulphites" (Christmann, 2001; Christmann and Freund, 2004; Flanzy, 2000 and Rankine, 2004).

3.5.3.2 Fining agent origin

Each fining agent used in this work will be detailed explained in the next chapter with every single fining agent and its allergy risks noted separately.

In Australia different research departments conducted an investigation to test if fined wines using potentially allergenic food proteins casein, milk, egg white, or isinglass, can provoke significant clinical allergy reactions (anaphylaxis) in patients with confirmed IgE-mediated relevant food allergies. They used a DBPC with Australian commercial wines fined using one or more of the legislation-targeted food proteins. Furthermore they analysed by blood basophile a larger panel for these wines. They concluded that the wines fined with egg white present "an extremely low risk of anaphylaxis to egg sensitive consumers." They write that even though milk protein-fined wine did not include anaphylaxis, there were insufficient subjects to determine statistically whether wines fined with milk proteins present a risk to the very rare milk-allergy consumers. And finally they summarize that in these wines the lack of basophil activation and anaphylaxis for the legislation-targeted wines according to good manufacturing practices suggests the insignificant residue levels of food allergens (Rolland *et al.,* 2006).
There are specific studies on this issue, especially concerning the residues in wine and methods of detection, they are mentioned throughout this work (Weninger and Görtges, 2005; Weber *et al.,* 2007; Weber *et al.,* 2009; Fischerleitner and Eder, 2006 and 2007; Fischerleitner *et al.*, 2012; Rolland *et al.* 2006 and 2008; Lacorn *et al.,* 2009 and 2011; EFSA, 2004, 2007a, 2007b, 2011a, 2011b; Christmann *et al.,* 2012; Wigand, 2008; Webber-Witt, 2009; Webber-Witt and Christmann, 2012; Deckwart *et al.*, 2012 and Deckwart *et al*., 2013)

3.6 Wine fining treatments

Part of the consumer quality requirements is due to the visual satisfaction. Turbidity is a negative factor in assessing a wine and it may possibly lead to customer dissatisfaction and economic loss to the winery. Limpidity and clearness is an essential factor of wine appearance and it also enhances the impression of quality on the palate (Lagune-Ammirati and Glories, 2002 and Lopez *et al.,* 2001).
Turbidity is the result of an optical phenomenon known as Tyndall-Effect and is caused by the presence of particles in suspension that deflects light from its normal path. The scattering of light in a wine depends on the size and number of particles in suspension. All wines are turbid in the beginning of their development. Haziness arises as a result of chemical equilibrium and physical changes in the wine solutes. The main factors responsible for it are the change of the pH-value ratios and the electric charge of the

colloids in the wine. Also substantially involved is the influence of oxygen, the redox ratios and suffered vibration in wine (Troost, 1988; Ribéreau-Gayon, *et al.* 2006). Wine substances can be divided in size as to be seen on Table 9.

Table 9 Wine substances divided into particle size

	Size (mm)	Example
Real solution	10^{-7}	Alcohol, sugar, acids and minerals
Colloidal solution	10^{-7} to 10^{-5}	Proteins and heavy metals complex
Insoluble particles	$> 10^{-5}$	Crystals, yeasts and bacteria

(Source: modified from Troost, 1988)

In wine it is common that most all of colloidal substances have a clear appearance, while suspensions make it turbid. Loss of clarity can come from three sources: microbial growth and production of polysaccharides; precipitation of chemical compounds and denaturation and complex formation between macromolecules, such as proteins, polysaccharides and polyphenolics (Troost, 1988).

It is imperative that the wine should be stabilised against unwanted changes prior to bottling. Those changes may be chemical in nature, or due to macromolecular interactions and changes in solubility. Microbial stability is also an important issue. Origin from turbidity can be from:

- Grapes, mash and must obtained by mechanical abrasion of plant, such as by pressing and pumping.
- Chemical modification, such as inorganic turbidity due to metal compounds, complex formation of colloidal nature, etc.
- Organic haziness, from proteins, nitrogen compounds, which normally are natural chemical colloids, protein and phenols expelling, aging haziness, that are reached through oxidation, warm haziness, cloudy pectin haze, tartrate crystals or colour matter (off-colour).
- Biological haziness trough yeasts or bacteria in wine.

3.6.1 Oenological use and aims of fining material

A fining practice in wine consists of adding certain substances into wine or most followed by settling or precipitation of the agent. Called *Collage* in French, *Schönung* in German and 'fining' in English, this is a winemaking procedure in which a product, either natural or synthetic, is added to the wine in order to remove unwanted material. A wine is fined in order to improve its appearance, to be clarified, to remove some off-flavour and to prevent some potential instability which may develop in the future by some wine elements. During the fining operation specific undesired wine constituents attach to the used fining agent either chemically or physically, to form a

new component that tends to separate from the wine either by precipitation and racking, centrifugation or filtration and are thus removed from wine. In summary reasons for the application of fining products are wine clarification, wine stabilization and taste improvement. Fining may also impact on the sensory quality of wines, the degree depending on the chosen agent and wine. Thus fining improves wine value (Margalit, 2004; Rankine, 2004 and Razmkhab *et al.* 2002).

3.6.2 Goals of fining practice

Main components to be removed in wine by fining practice

- Phenolic compounds, polymerised phenols, tannins and anthocyanins; soften wine by reducing bitterness and astringency, thus colour.
- Protein: fount of turbidity – haze-forming potential
- Off-character or off-character-forming potential
- Metal ions

Historically, various substances such as egg whites, blood and milk have been used as finings (Nikel, 2009). These are still used by some producers, but more modern substances have also been introduced and are more widely used. In German winemaking books from 1872 and 1908 there are references about the use of milk and egg white as fining material. In the oldest book they indicate the use of fresh milk for white wine, mentioning that it “decolourize” red wines. In the second book there is a mention that casein had been used already in that period for a long time. Its recommendation is against off-flavours, and as a fining agent is indicated as very good for the reduction of colour matter if carefully used, because it “attacks wines bouquet and can overly reduce the colour”. The quantity of milk to be used is indicated from 1 to 2 litres per hectolitre. Egg white is indicated uniquely to be used as reducing tannins, with the goal of being used in very tannic wines, and quite logically, only for red wines, namely in France. The quantity indicated is from 2 to 4 eggs, only the white, per hectolitre. There is an old picture (Figure 8) from 1529 with a man working in the cellar mixing a fining agent to prove how old is the usage of fining agents on winemaking practices (Babo, 1872 – page 222 to 227 and Windisch, 1908 – page 364 to 367).

Figure 8 Old drawing with a man fining a wine (1529)

(Source: Troost, 1988 –page 356)

The correct time to fine depend on the winery conditions, i.e. temperature, as well as on the wine's acidity and pH. The less acidic a wine, the higher the rate of colloids in suspension; which are responsible for hazes. While in more acidic wines hazes are settled out much quicker. An early treatment applied to a young wine has advantage over a finished and equilibrated one that normally reacts in a more unfavourable manner. There are many other factors such as air contact during wine-making process and racking, the amount of tannins, pumping shocks and movements which wine is exposed to that has an influence on the time of fining and its relation to wine turbidity. It is always advisable to fine before filtering (Troost, 1988 and Gaelle *et al*., 2009).

Fining mechanisms can be quite complex and the simplest explanation proposed by Rankine (2004) is the one of electrostatic attraction, whereby the fining carrying a particular electric charge reacts with wine constituents carrying the opposite charge and the neutralized combination that forms precipitates out. Protein fining agents show a positive charge at wine pH, depending on the Ip of each protein and generally react with polyphenols by forming bonds between the phenolic hydroxyl and the peptide bonds of the amino acids. Large polyphenols such as tannins and polymerized anthocyanins are removed. Fining removes those tannin molecules that respond most readily with proteins, and are the most aggressive from an organoleptic point of view (Rankine, 2004).

Fining agents operate by taking advantages of hydrophobic or hydrophilic interactions between the agent and the species to be removed (Würdig and Woller, 1989).

Troost (1988) lists some of the scientific bases related to winemaking practices, which are important to be mentioned here, concerning the basics rules of fining.

1. The sediments, generally flocks, have to have a superior specific weight than the wine, otherwise it do not sediment. These sediments caused in wine due to

the haze and the solid products are dissolved or as a colloid, which is in reality what produce the cloudiness.

2. The wine to be clarified might be quiescent, mainly biologically.
3. Wines colloids are charged positively or negatively. Fining material must have an opposite electrical charge to be able to flocculate these colloids.
4. The more acidic is the wine (lower pH) the better the clarification occur and the lower the amount of fining material required.
5. For a successful fining the quantity and concentration of material plays an important role. It is always better to find the lowest possible dose required to achieve efficiency.
6. The effect of a clarification also depends on the temperature in which it is carried out.
7. The effectiveness of fining is dependent on how the product is prepared and added. The speed of application and the regularity of fining distribution are determining factors.
8. The process of clarification process varies with time. This time will be greater:
    a) the greater the distance the flocculants have to travel to settle out (this is the tank height),
    b) the greater the resistance exerted on the flocculants in wine, particularly when the flocks are big
    c) the greater is the viscosity of a wine.
9. The sequence of addition is important for a successful fining when more products are added at the same time.
10. The practice of fining has low influence on turbidity caused by microorganisms, like yeast and bacteria.
11. When choosing the products, only products that are completely eliminable from the wine and that do not influence in an unfavourable way the wine taste and colour should be applied.
12. The surfaces of the barrique or tank within which the fining occurs should be as flat as possible inside. Tanks might be more effective than wood-vessels.

Recent studies show that every fining agent will act differently depending on the level of polymerisation of phenols. Furthermore not only the type of protein fining agent but also the size (kDa) will determine the particles in which it will interact. The author of this study suggests that, therefore “the oenologist’s choice of protein fining agent for clarification and for the reduction of particular phenolic compounds is important and should be very carefully considered” (Cosme *et al.*, 2009).

Some wines can achieve a brilliant and clean condition just by settling – without fining agent help, but it normally takes a considerably long time. Furthermore just settling is sometimes not enough to ensure the product stability. Periodically a new formation of haze is triggered after racking or other wine operations. This natural phenomenon is part of the aging of wine.
To fine the wine it is important to know the reason(s) why it might be treated. Knowing the reason it is easier to decide which treatment to be undertaken. It is important to use the minimum quantity of finings, which will carry out the task when fining wines – as mentioned wines fifer in their composition and the amount of a particular fining required. Therefore a trial fining is necessary before the actual fining in the winery (Rankine, 2004).

3.6.3 OIV Guideline for good fining practice

Of relevant importance to be mentioned at this work is the OIV Guideline of good fining practice for wine to be applied after the use of proteinaceous (allergenic) wine fining agents - casein and egg white (OIV Guideline, 2013). This is a published working document. Here are the steps indicated on this document:

"1. Fining agents shall be free from undesirable taints and must conform to all applicable regulations. They should be stored in a cool, dry environment in sealed containers, or in other recommended storage conditions as advised by the manufacturers.
2. It is strongly recommended that laboratory scale trial runs be conducted prior to treatment of wine in the winery.
3. The laboratory trial runs should also duplicate, as far as possible, the treatment to be conducted in the winery, giving attention to the batch of fining agent to be used, the method of its preparation and addition to the wine, and the temperature of the laboratory sample with respect to that of the bulk wine to be fined. Hydration protocols for protein fining agents should be consistent between laboratory and winery.
4. A minimal volume of distilled, de-ionised or other potable water should be used in order to dissolve or disperse the fining agent without overly diluting the wine (applicable regulations must be met).
5. The quantity of fining agent used should always be the smallest amount needed to achieve the desired result as stipulated by winemaker sensory and/or analytical evaluation, and in no case shall the amount used exceed any recommended typical addition rate.

6. Thorough and adequate mixing of the fining agent into the juice or wine should be ensured, and sufficient time should be allowed for the material to react prior to immediate racking and/or subsequent filtration.
7. Industry recognized best practice filtration methods (including passing the wine through a fine powder filtration process and/or pre-bottling filtration through a 0.65 µm or smaller membrane filter, or performing treatments of equivalent effect) should be used to remove insoluble protein fining agents. Where the treated wine is simply racked off the lees remaining from the fining treatment and bottled without filtration, or where a less rigorous filtration or other technique for removal of the lees is applied, an analysis must always be conducted at some stage prior to bottling. However, it is recommended to conduct analysis of filtered and unfiltered wines to confirm that no residue fining agent(s) can be detected.
8. Routine, periodic monitoring of the fining process shall be conducted. In general, this will entail analysis of a sample of fined wine using a sufficiently sensitive method of analysis for the fining agent in question. The frequency of sampling should be adequate to give confidence that the fining processes are being conducted in such a way as no detectable residue of fining agent remains in the treated wine. Corrective action must be taken where the analysis of such wines indicates the presence of residue fining agents, or the product labels must reflect that presence.
9. Verification should be conducted at regular intervals, and should consist of a review designed to ensure that monitoring is occurring carefully and consistently, at a frequency that is adequate to give confidence that the fining processes are being conducted in such a way as to leave only undetectable fining agent residues. Verification should also ensure that adequate and timely corrective actions are taken where evidence is obtained that indicates the potential for the presence of residue fining agents in a treated wine (e.g. through false positive results).
If the fining guidelines above have been respected, it has been established from scientific studies that no residue fining agents will be detected in the wine”(OIV Guideline, 2013).

3.6.4 Fining agents of organic origin

There are many fining agents of organic origin used in wine. Some of them are no longer used whereas others are applied only in some countries. Namely blood, wine yeasts, mannoproteins, chitosan, seaweed and tannins, etc. In this work only fining agents applied in this study will be described, in which an allergic potential is identified or if the fining agent may be used as an substitute – non allergenic (Troost, 1988; Ribéreau-Gayon *et al.*, 2006; Bornet and Teissedre, 2008 and Cabelo-Pasini *et al.*, 2005).

3.6.4.1 Egg origin

Egg consists of a porous carbonate shell, yolk, and albumen commonly known as egg white, this last part is the only one used in winemaking. Egg white is formed by 86.6% water and it contains over half of the proteins in egg (~ 9.7-10.6% protein by weight), but are low in lipids at 0.01%. Over 24 diverse proteins have been identified and isolated from egg white. The main proteins comprise ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), and lysozyme (3.4%). The following table lists selected properties of the major egg white proteins Table 10 (Mine *et al.,* 2006 and Mine, 1995).

Table 10 Properties of egg white proteins

Protein	% protein in egg white	Molecular Weight (kDa)	pI (pH)	Denaturation Temperature (°C)
Ovalbumin	54-58	44.5	4.5	84.0
Ovotrasferin	12-13	77.7	6.1	61.0
Ovomucoid	11	28.0	4.1	77.0
Ovomucin	1,5-3,5	5.5-8.8 x10^3	4.5-5.0	Unknown
Lysozyme	3,4-3,5	14.3	10.7	75.0

(Source: modified from Mine, 1995 and Belitz *et al.*, 1982)

There are many different forms of egg agents that may be used for wines (Lagune-Ammirati and Glories, 2001).Here are the main two:

- **Pure fresh hen egg white:** the process is to break the eggs and separate the yolks. Very often the egg whites are made into a solution of sodium chloride – table salt (0.5%-0.9%). The reason is because this solution assists in dissolving the protein, since it upholds the globulins in solution. The quantity to be used varies from one to three eggs per hectolitre. Excessive mixing should be avoided as this will lead to significant foaming. Some winemakers use frozen egg white, which produces similar results to those obtained using fresh egg white (Rankine, 2004; Troost, 1988; Margalit, 2004 and Ribéreau-Gayon, 2006).
- **Dried powder of egg white:** also called ovalbumin or (dry) ovalbumin, since it is obtained from the drying of egg whites. This powder (also in flakes form) is comprised largely from one protein, which is ovalbumin. Many commercial powders of ovalbumin are known not to be pure of ovalbumin protein. They enhance other egg proteins in addition to ovalbumin (EFSA, 2011a). It is also available commercially in ready-to-use, sterilised, liquid form. Its colour varies from white to golden yellow. This powder is perishable and cannot be kept

opened for long periods in the winery, neither is it recommended to warm it, since egg ovalbumin may be spoiled by heat. Usage consists on dissolving the powder into some water. About three grams of dry powder is equivalent to one egg. The quantity often used is from 4-16 g per hectolitre. To apply into wine, both; fresh or powder, should be stirred very well. The wine can be racked after one week (Rankine, 2004; Troost, 1988; Ribéreau-Gayon, 2006 and Margalit, 2004).

The Ip of ovalbumin is 4.5 and its molecular weight (MW) is 44.5 kDa (Mine, 1995 and Belitz *et al.*, 1982).

Known to be the oldest fining practice in oenology, egg fining is undertaken in red wines, used to clarify (i.e. fining) and remove undesirable substances such as proteins, phenolic and harsh tannin compounds that cause bitterness and astringency (Rankine, 2004; Troost, 1988; Ribéreau-Gayon, 2006 and Margalit, 2004). It has some discolouring effect and makes bitterish wines milder, therefore not indicated for light red wines (Troost, 1988 and Ribéreau-Gayon). It is recommended for the polishing and the softening of red wines. This fining agent protein interacts mainly with higher polymeric phenols rather than lower ones or monomers (Margalit, 2004). A study showing the effect of fining agent on different components of wine, showed that the oligomeric flavan-3-ol was extensively decreased by egg ovalbumin, but did not lower the monomeric flavan-3-ol significantly, or that ovalbumin reacts more with proanthocyanidins that has a higher level of polymerisation (Cosme *et al.*, 2009). Ribéreau-Gayon *et al.* (2006) has already reported that monomeric flavanols as well as dimeric and trimeric procyanidins are not affected by fining. According to Siegrist (1996), variations in volatile compounds due to fining are on the order of 8% for egg ovalbumin (Ribéreau-Gayon, 2006).

Egg white ovalbumin is positively charged and will attach and absorb negatively charged substances such as tannins to form a flocculent precipitate in wine. From a colloidal point of view it does not flocculate a great deal, but precipitates a firm deposit. The elimination of precipitate is carried out by decanting, racking, and may include centrifugation or filtration prior bottling (Ribéreau-Gayon, 2006 and Jakob *et al.*, 1997).

Allergy importance related to wine

The occurrence of allergy to egg proteins and above all to ovalbumin among the common population has been reported to be around 0.3% in adults. EFSA reports that based on the available statistics from a national food consumption survey carried out in Australia (Australian Bureau of Statistics, 1999), it is assumed that the interviewee has the highest wine consumption rate of approximately 1L/d. Assuming a residue

concentration of 1µg/L of ovalbumin in wine, this would correspond to a daily intake of 1 µg ovalbumin. The applicant states that this level is underneath the lowest dose eliciting adverse reactions in egg allergic individuals as identified in a number of challenge studies. Literature research was also presented by the applicant showing no recorded reaction to egg ovalbumin in the past 15 years in the Australian Wine Research Institute. Yet the EFSA panel notes that "under-reporting of reactions caused by egg proteins after ingestion of wine may have occurred since consumers and health professional may not be aware that egg white products are used in winemaking process" (EFSA, 2007a page 4 of 7). The EFSA panel therefore considered that wines fined with ovalbumin may trigger adverse reactions in susceptible individuals under conditions of use stated by the applicant (EFSA, 2007a).

Later application for permanent exemption from labelling to EFSA, in 2001 from the European Commission (EC) by OIV and NDA on ovalbumin/egg white has another published scientific opinion. The Panel consider in their opinion that methods used for detection of ovalbumin have been improved, but there was not sufficient information provided by the applicant about the levels of residue ovalbumin or other egg allergens which may be found in commercial wines fined with egg ovalbumin. Again the Panel opinion concludes that wines fined with ovalbumin/egg ovalbumin may trigger adverse reactions in susceptible individuals (EFSA, 2011a).

3.6.4.2 Lysozyme

In oenology lysozyme is not defined as a fining agent, but as an additive and it is included in this chapter as it is an enzyme present in hen egg white, as shown on Table 10. Lysozyme is a natural occurring enzyme (Christmann, 2001).

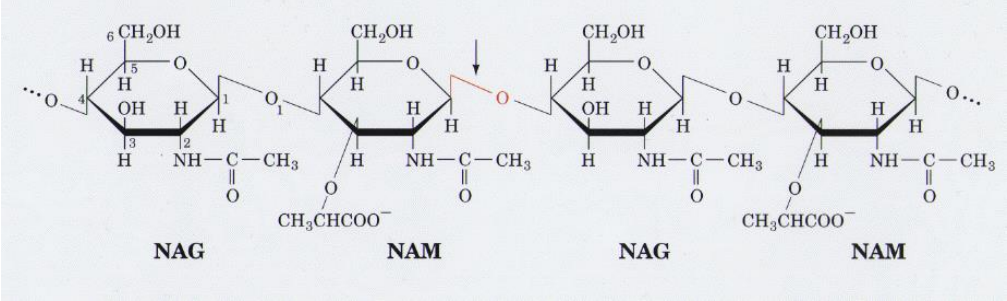
Numerous methods are used in laboratory practice to separate lysozyme from hen egg white, but only some of them have been used in the industry. Separation methods are not simple; therefore lysozyme is a costly agent if compared with other aid material used in winemaking. Methods to separate lysozyme comprise its straight crystallization from egg white. These techniques are based on the phenomenon of the adsorption of the enzyme by certain substances, chromatographic techniques, or membrane techniques, particularly ultra-filtration (Cegielska *et al*., 2008). Commercially lysozyme for wine is found in the form of a dry white fine powder.

The Nobel Prize winner Alexander Fleming, who discovered penicillin, accidentally discovered also lysozyme. Nasal secretion of a patient was used for his experiment on bacterial culture. These cells were lysed, and therefore the origin for the name lysozyme: lyse + enzyme (Fleming, 1922). Lysozyme is a bacteriolytic enzyme commonly found in nature, apart from egg white it can be found in different tissues and secretions of animals and in the human body.

Lysozyme (E.C.3.2.17, N-acetylmuramide-glycohydrolase) is a small protein found as a single polypeptide chain of 129 amino acids, in which lysine is the N-end amino acid and leucine is the C-end one. It is characterised MW of approximately 14.4 kDa and a pI of 10.7-11 and its maximum activity is at 4-7 pH (Christmann and Freund, 2004). It contains four disulphide bridges (S-S), providing its high thermal stability of the enzyme. The enzyme molecule is a solid complex in the form similar to an ellipsoid with dimensions of 4.5 x 3.0 x 3.0 nm (Cegielska *et al*., 2008 and Christmann, 2001).

In wine lysozyme is used to prevent MLF, due to its lytic enzymatic activity against gram-positive bacteria, such as lactic acid bacteria. Gram-negative bacteria and yeasts are not attacked by lysozyme lytic effect; they have a different cell wall composition than gram-positive bacteria (Christmann and Freund, 2004; Dietrich, 2005 and Cunningham, 1991). Lysozyme is very substrate specific (Figure 9) hydrolysing cell walls' beta-1,4-glycosides bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan resulting on a loss of the cell wall strength that ends in cell bursting (Dietrich, 2005).

Figure 9 The polysaccharide substrate of lysozyme



(Source: http://www.nd.edu/~aseriann/lysosubst.gif)

Lysozyme is mostly used in white wines to avoid MLF, to maintain wine freshness and avoid other aromas coming from MLF that in a number of wines are undesirable, but may also be used in reds. Besides it can reduce the requirement for SO_2 and decrease biogenic amines (Roure, 2005). Some wine substances happen to reduce lysozyme activity; tannins, colour matter and bentonite from fining treatment. It is well known that bentonite removes proteins (Gerbaux, 1997 and Dietrich, 2005). Due to relatively high amounts of protein that comes into the wine by lysozyme, protein stability tests must be made prior to bottling (Dietrich, 2005).

Although lysozyme is already used for longer time in other industries, such as by the cheese industry, it entered late in the wine industry - around the "90s" (Chinnici, 2005). In 1997 its use is a practice accepted by OIV and as such is one of the additives used during the winemaking process (Resolution oeno, 1997). There is a legal limit to be added in wine determined by the EC No 1493/1999 and 2066/2001, which is when

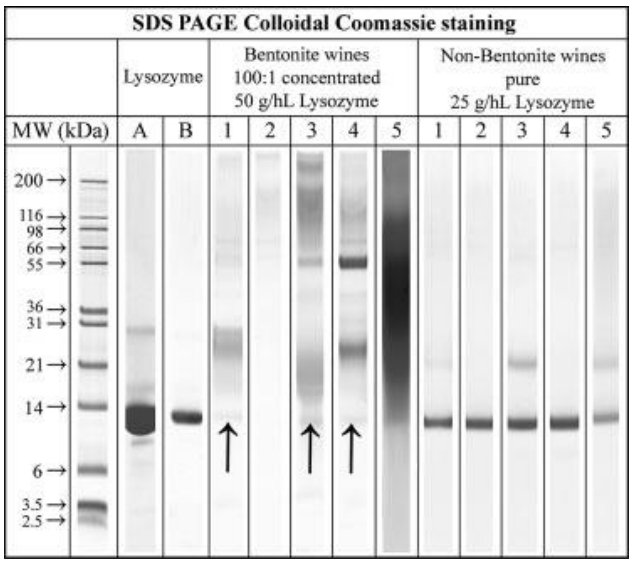
must and wine are treated with lysozyme. The accumulated dose must not exceed 500 mg/L (50 g/hL) (Resolution oeno, 1997). A study shows that an amount of 125/250 mg lysozyme/L is sufficient to stabilise wines after malolactic fermentation (MLF) when bacterial counts approximate 10^6 cfu/mL (Gerbaux, 1997).

Allergy importance related to wine

Allergic reactions exclusively to lysozyme were reported by numerous authors (Pérez-Calderón, 2007; Malmheden, 2004; Frémont, 1997, Camp, 1988). Due to this well-known allergenicity of lysozyme it must be declared on the wine label if present in the final product not only within the EC, but in other countries such as Japan, Oceania countries and the United States of America. The European Directive related to lysozyme is by Annex IIIa in Directive 2007/68/EC amending Directive 2000/13/EC (EU-Richtlinie 2007/68/EG).

There are some factors to be considered according to recent studies done by Weber *et al.* (2009). Various results were acquired regarding the amount of lysozyme in wines and their *in vitro* and *in vivo* reactivity in patients allergic to hen's egg. The amount of lysozyme found is reliant on the quantity added, the wine variety, colour and also on fining with bentonite as to be seen on SDS-PAGE of Figure 10 with colloidal Coomassie staining of lysozyme-treated wines. (A) Lysozyme adjuvant for wine treatment; (B) analytical grade lysozyme. 1, Riesling Mosel; 2, Riesling Rheingau; 3, Pinot Blanc; 4, Pinot Gris and 5, Dornfelder Rheinhessen.

Figure 10 SDS-PAGE of lysozyme-treated wines



(Source: Weber *et al.,* 2009)

The study shows that even though the quantity of lysozyme in bentonite treated wines appear insignificant, non-bentonite-treated wines contain lysozyme, eliciting

immunological reactions in egg allergic patients *in vitro* and *in vivo* (Weber *et al.*, 2009). In the same studies he explains that wines treated with lysozyme require a higher amount of bentonite. Consequently this protein is almost removed, but small residues of lysozyme between 0.001 and 0.006 g/hL were detected in wines treated with 25 and 50 g/hL of lysozyme. He highlighted that even in red wine - Dornfelder, it was possible to presence the remaining lysozyme. If lysozyme is applied on a legal limit according to the EC, and consequently fined with bentonite, all results in his study showed a negligible risk *in vitro* and *in vivo* by SPT. Different and opposite results are found by wines non-bentonite fined, even in red wines, that are known for their natural protein-precipitating property, clearly pose a risk for allergic consumer based on *in vitro* and SPT results. Weber as well indicates the use of meta-tartaric acid as an adsorbent of lysozyme that should be considered as lowering its content in wine, as an alternative to bentonite fining. Weiand (2004) has also reported that metatartaric acid precipitates lysozyme (Weber *et al.*, 2007; Weiand, 2004 and Weber *et al.*, 2009.

3.6.4.3 *Milk origin*

Milk is a liquid taken from the mammary glands of female mammals. Today merely dairy cow's milk is meant as a commodity (Belitz *et al.,* 1982). In this work when milk is referred it only refers to cow's milk.

In milk there are two major categories of dispersed proteins that are broadly defined by their chemical composition and physical properties. The casein family contains phosphorus and will coagulate or precipitate at pH 4.6. Whey is the serum protein family of milk and consists of roughly 50% ß-lactoglobulin, 20% α-lactalbumin, blood serum ovalbumin, immunoglobulins, lactoferrin, transferrin, and many minor proteins and enzymes. Alike to the other key milk components, each whey protein has its individual characteristic composition and variations. In wine the use of whey as a fining agent is new and not very common. Whey proteins do not contain phosphorus, and these proteins remain in solution in milk at pH 4.6. The principle of coagulation, or curd formation, at reduced pH is the basis for cheese curd formation. In cow's milk, approximately 82% of milk protein is casein and the remaining 18% is serum. Particles of varying size, which are known as micelle and mainly consist of calcium salts of casein. There are various other proteins solved in the milk serum, as well as carbohydrates, minerals, fats and other ingredients. The main difference between casein and whey is that the second one is soluble in acidic medium as for caseins flocculate in acidic solution (Belitz *et al.,* 1982 and Weber *et al.* 2007).

Casein exists in the milk in an aggregated form known as casein micelles. These show some resemblance with surfactant-type micellae in a sense that the hydrophilic parts reside at the surface. Caseins are characterised by sequences of hydrophobic and hydrophilic amino acids, resulting in an ambiphilic character, thus micelle-forming properties. In milk, caseins are negatively charged whereas in wine they have a net

positive charge. The casein component of milk is relatively heat-stable, capable of surviving pasteurisation at ~62-71 °C. In opposition the whey protein component is denatured at these temperatures (Horne, 2002 and Weber *et al.,* 2007).

The caseins (from Latin *caseus* "cheese") are a family of hetero phosphorylated proteins. There are four different types of casein proteins: αs1-, αs2-, β-, and κ-caseins held together by calcium phosphate bridges on the inside (Table 11) and each has its own amino acid composition, genetic variations and functional properties; all of them with low MW, less than 30 kDa. These casein micelles also transport a large proportion of the minerals, such as calcium and phosphate. Three affiliates of the family, α -, S1 aS2- and β -casein precipitate in the presence of millimole levels of ionic calcium. K-casein does not precipitate and in mixtures with the other caseins inhibits the precipitation reaction, forming colloidal stable entities instead (Horne, 2002).

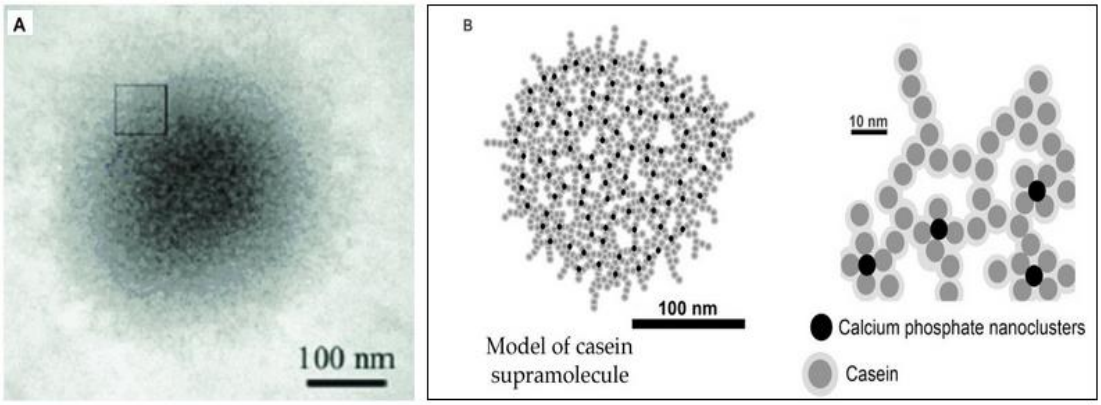
Table 11 Several genetic variants of bovine casein

Type	Molecular weight (Dalton x 10^{-3})	pI
α-s1	22-23	4.2-4.7
α-s2	25	4,1
β	23-24	4.6-5.1
κ	19	4.1-5.8

(Source: modified from Belitz *et al.*, 1982)

Casein consists of a reasonably high amount of proline peptides, which do not interact and gives it a relatively random open structure, due to the amino acid composition. There are also no disulphide bridges. As a result, it has a relatively small tertiary structure and cannot easily denature. Casein is not coagulated by heat. It is precipitated by acids and by rennet enzymes, a proteolytic enzyme typically obtained from the stomachs of calves. It is fairly hydrophobic, making it poorly soluble in water. Casein solubility is pH dependent and is also affected by ionic strength and composition. The caseins in the micelles are held together by calcium ions and hydrophobic interactions as in this model shown in Figure 11 (Sigma-Aldrich, 2012).

Figure 11 Electron micrograph (A) and model (B) of a casein supramolecule



(Source: McMahon, 2012)

Casein is readily dispersible in dilute alkalis and in salt solutions such as sodium oxalate and sodium acetate. Therefore casein is made alkaline before being added into wine. This procedure consists of adding potassium or sodium bicarbonate or carbonate, or possibly potash in a solution for better dilution. The casein proteins have regions of net negative charge due to the fact that they are phosphorylated. These regions can undergo charge interactions with positively charged forms in the wine. Caseins are insoluble at low pH, thus the pH of wine explains why it precipitates. The proteins have also hydrophobic or non-polar regions that are exposed when caseins denature at wine pH. These regions can interact with phenolic compounds and other components. Finally, most proteins will have a net positive charge at wine pH due to the pKa values of the amino acid side chains (Horne, 2002 and Fox *et al.,* 1998).

Casein in association with sodium or potassium ions forms a soluble caseinate that readily dissolves in wine. In wine the salt dissociates and insoluble caseinate is released. Many types of milk fining materials may be used - skim or whole milk, lactic casein, sodium caseinate and whey proteins. Sodium caseinate prepared by precipitation of casein micelles by reducing milk pH to 4.6. It also finds wide use in the food industry as an emulsion stabilizer.
In the wine industry and for European legislation this material has been accepted in 1978 by Regulation 1861/78 EEC, but has been used in many countries already before this date. (Horne, 2002; Ribéreau-Gayon *et al.*, 2000 and Millies and Reimerdes, 1992).

Casein proteins are in employment in large amounts in wine production in many countries. In Germany up to 20% of the wines are fined with casein after the German institute (Deutscher Weinverband, 2010). While in France over 20.5 million hectolitres of wine are fined with casein annually, which corresponds to around 40% of French wines (Weber *et al.*, 2007).

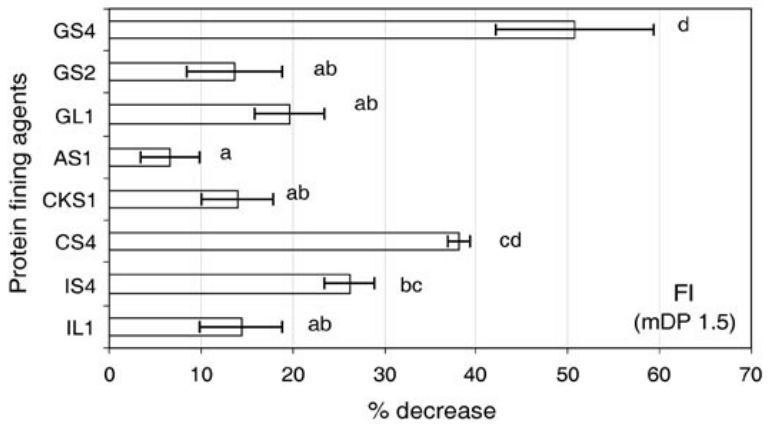
- **Whole milk** can be effective in certain cases. It improves the colour of wine and reduces mouldy odours. The effect in the treatment is due to the fat content of milk. Skimming reduces the absorption capacity but increases the clarifying effect. Fining with whole milk is not permitted in the EU. In one litre of milk there is around 30 g of casein and 10-15 g of other proteins. If whole milk is added to wine (at rates up to 1 L per hectolitre) the cream tends to float on the surface, and for this reason skim milk is more frequently used. It is simply mixed with water and slowly added to the wine by mixing. (Rankine, 2004) Skim milk is gentler in its action than the casein products (Ribéreau-Gayon *et al.,* 2000). Although milk fining is an old and well known practice, this work concentrates on milk proteins used in wine stand of milk as a whole (Windisch, 1908 and Babo, 1872).
- **Lactic casein** is the preferred form by many winemakers, but it is not overall accessible and is not always the same. Commercially available caseins for example are very different and the type of production affects the nature and purity considerably (Würdig *et al.* 1989). It is found commercially in powder form, which is gained from coagulated skimmed milk. To dissolve it needs to be made alkaline (pH 11) and this is achieved by dissolving in warm water with about one third of its weight of potassium carbonate. The normal dose is from 5 to 20 g/hl, although in curative treatment 50 g/hl or more may be used. Some sources indicate up to 80 g/hl. Casein powder's preventive action is not fully understood, but it affects phenols, either by eliminating them or, more probably, by protecting them from oxidation. (Ribéreau-Gayon *et al.,* 2000 and Troost, 1988).
- **Potassium and Sodium caseinate**. Potassium caseinate is water soluble and is favoured for this purpose. Sodium caseinate is normally not used because it increases the sodium amount in wine (Moreno-Arribas, 2009). These caseinates are already alkaline, so is more soluble but still somewhat difficult to dissolve. Accordingly, potassium carbonate is added to the warm aqueous sodium caseinate solution, which is then stirred and left overnight. The rate of addition is the same as for lactic casein (Ribéreau-Gayon *et al.,* 2000). Sodium caseinate may also be found under the commercial name *Vinpur-special*, which is a compound including cellulose to be directly applied to the wine. It is indicated for wines or musts that have passed through harsh treatments, such as screw pressing, harvesters or decanters. In other words, wines with high content of tannins (Milles and Reimerdes, 1992 and Erbslöh, 2012).

- **A whey protein is** used as a powder. It has good solubility in water and in wine. The amount to be applied is similar to above-mentioned proteins or slightly over this quantity (Meinl, 2010).

All milk related fining agents are described as used mainly for colour and flavour enhancement of high-coloured white wines. This favourable fining agent for white wines has a "refreshing" effect on their colour and flavour; it is a traditional treatment for white wines to remove phenolic bitterness, for softening the palate, removing harshness and off-flavours, and for lightening the colour. The decolourising power of different caseins is directly related to their formal titration values, a measure of free amino acid groups. This fining is broadly used in Sherries. It may also be used preventively and curatively on yellowing and maderisation of white wines (Würdig and Woller, 1989; Troost, 1988; Ribereau-Gayon *et al.*, 2000; Flanzy, 2000; Margalit, 2004 and Rankine, 2004).

There is a recent and detailed study on the effect of various proteins on different MW proanthocyanidin fractions of red wine during fining. This study shows that casein reduces around 35% of the monomeric falvan-3-ol, which is generally associated with bitterness, whereas potassium caseinate reduces around 15%. On the contrary of ovalbumin, this study shows that casein is more likely to react with monomeric proanthocyanidins, as to be seen on next figure taken from this study (Cosme *et al.*, 2009).

Figure 12 Decrease of the tannic fractions (%) with mDP of 1.5



Decrease of the tannic fractions (%) with the mean degree of polymerization (mDP) of 1.5, after fining treatment with distinct proteins. Concentration of condensed tannins in unfined wine: FI (15.1 ± 0.7). Error bars represent the standard deviation. Means within a column followed by the same letter are not significantly different (LSD, 5%). (Isinglass (IL1), isinglass (IS4), casein (CS4), potassium caseinate (CKS1), egg ovalbumin (AS1), gelatine (GL1), gelatine (GS2), gelatine (GS4).)

(Source: Cosme *et al.*, 2009)

After the addition of casein to wine it speedily flocculates. The flocculation occurs exclusively due to the acidity of the medium. This casein property is together positive because it does not produce overfining, but also negative due to the fact that this fining agent is rather difficult to use or it can also lead to incomplete fining. It must be quickly dispersed through the complete mass of wine before it flocculates, which occurs in a very short time. An injection pump is the best solution, making it possible to avoid losing any of the fining agents through partial flocculation before it is completely dispersed in the wine (Ribéreau-Gayon *et al.*, 2000). Another negative aspect of casein fining may be the tendency to strip the wine of aroma and flavour (Bisson, 2013). The presence of tannins (added) is positive since they precipitate and clarify the wine, as it improves its clarification (Cruess, 1963). Troost (1988) indicates the use of casein or sodium caseinate alone or just before addition of gelatine.

The addition of lactic casein or sodium caseinate should be made slowly, preferably through a venture valve on the inlet side of a pump circulating the wine in the tank. After the fining has settled the wine is racked or filtered. If the fining is added too quickly it coagulates and either floats to the surface or settles to the bottom as lumps, both of which are largely ineffective. In order to save on handling operations casein fining may be used in conjunction with and before other treatments, such as cold stabilization or bentonite.

Allergy importance related to wine

A few studies have been done concerning the allergenicity of milk proteins in wine, not only in experimental but also in commercial wines, from Europe and overseas (EFSA, 2011b).

Weber *et al*. (2009) reported results of 32 experimental and 61 commercial white wines with ELISA test. The authors agreed that allergic reactions due to the consumption of casein-treated wines cannot be expelled. EFSA observes from Weber studies that the lowest dose of casein capable of triggering an allergic reaction in a sensitive person is vastly unsure. Yet it shows that reactions have been expressed to a dose of 300 mg of low-fat milk powder, which corresponds to approximately 105 mg of milk protein or 90 mg of casein (Lam *et al.,* 2008). Also some case reports propose considerably inferior doses as capable of triggering reactions in extremely sensitive persons. Therefore final conclusion of EFSA' Panel on casein/caseinate/milk is that these products may trigger adverse reactions in susceptible individuals under the conditions of use proposed by the applicant (EFSA, 2011b page 8).

3.6.4.4 Fish origin

Isinglass (*cola piscium*) is a protein obtained from swim bladders of tropical fish by solubilisation in organic acids and consists predominantly of the protein collagen - around 70%. Different species of fish (kingfish and thread fish of Saigon and Penang, and the catfish of Karachi and Brazil) are used for isinglass extraction; even Sturgeon has already been used for centuries. Isinglass is broadly used commercially and historically to clarify alcoholic beverages by aggregation of the insoluble particles and yeasts contained in it. The German word for Isinglass is *Hausenblase*, "*Hause"* from Huso (sturgeon fish) and *blase* from bladder. Troost (1988) reports the year of 1517 for winemaking by monks of Eberbach Abbey in the Rheingau region and pointed out that the way it is used nowadays is pretty much similar to that period (Hickman *et al*., 2000; Würdig and Woller, 1989 and Troost, 1988, page 363). Some oenological isinglasses are made by other fish parts, due to price of production (Ribéreau-Gayon *et al.*, 2006)

This fining agent is present as a rod-like triple helical molecule and presents thermal lability. Thermal denaturation of isinglass occurs at 29°C and it is no longer efficient as a clarifying agent, therefore the collagenous triple helical configuration must be maintained. The rod-like structural integrity of the collagen triple helix is subsequently essential for efficient clarification (Hickman *et al*., 2000).

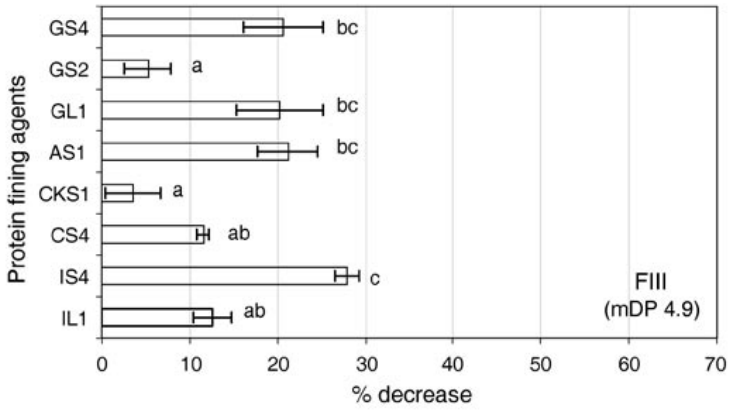
The molecular weight is about 140,000 and the Ip is in the range 5.5-5.9. The Ip of isinglass in aqueous solution was found to be 5.9 (Freundlich and Gordon, 1936). According to Rankine (2004) isinglass Ip is 5.5, so that it has a net positive charge at wine pH, which electrostatically attracts negatively charged particles in wine (Margalit, 2004 and Troost, 1988).

Isinglass is supplied to the industry as a fine powder, which should be opalescent, odourless, yellowish and shiny, without having meat residues or rust stains. Further forms of isinglass found are a paste, strips, sheets, whitish chips or as a highly viscous liquid. These forms may be added directly to the wine or should first be made into a solution and then added to the wine to cause aggregation of insoluble particles, which then sediment to the bottom of the container or can then be removed by filtration. During addition it should be stirred, depending on the size of vessel, for approximately 15-30 minutes. The optimal temperature to fine the wines with isinglass is from 18-20°C. Whereas temperatures over 25°C are not recommended, since it influences the precipitation and coagulation of this fining agent. The indicated amount to be used is 0.5 to 2.5 grams per hectolitre. After its addition the wine clears, depending on storage temperature and barrel size, in a few days and can be racked off after 8 to 10 days. Thus after 2 to 3 days the haze has normally settled. Silica sol used together with isinglass is indicated as a helping method on fining flocculation (Hickman *et al*., 2000; Jakob *et al.*, 1997, Ribéreau-Gayon *et al*., 2006 and Troost, 1988).

Isinglass uses in oenology are indicated specially for clearing white wines with low phenolic content, because phenols and colour matter are barely removed by this agent. The material interacts mainly with monomeric phenols rather than polymeric ones. Moreover, it removes slight coarseness or off-flavours in wine taste (Jakob *et al.*, 1997). According to Rankine (2004) isinglass removes phenolics and bitter tannins. He claims that isinglass acts without unduly removing colour, as may occur when casein or gelatine are used, since it removes more leucoanthocyanins a less condensed tannins than gelatine and casein. Ribéreau-Gayon *et al.* (2006) describes isinglass as an enhancer of brilliance and that it reinforces the yellow colour of white wines. This fining agent is known to have a soft action to wine and may be used even before bottling. It has been the most used fining agent in the Mosel region wines in Germany for a long time, as their wines are known to be really acidic. Troost also reported isinglass in 1989 as a low-cost fining agent (Margalit, 2004; Troost, 1988 and Rankine, 2004).

A recent and detailed study by Cosme *et al.* (2009) on the effect of various proteins on different MW proanthocyanidin fractions of red wine during fining, shows that isinglasses reduces up to 28% of the polymeric falvan-3-ol, which is generally associated with astringency. On this study Figure 13 two different isinglasses where used; fish-skin and swim-bladder isinglass, they reduced 13 and 28%, respectively. The second isinglass is two times more efficient than the first in this study. Monomeric flavan-3-ol reduction by isinglasses can be seen on Figure 12 (Cosme *et al.*, 2009).

Figure 13 Decrease of the tannic fractions (%) with mDP of 4.



Decrease of the tannic fractions (%) with the mean degree of polymerization (mDP) of 4.9, after fining treatment with distinct proteins. Concentration of condensed tannins in unfined wine: FIII (582.2 ± 57.6). Error bars represent the standard deviation. Means within a column followed by the same letter are not significantly different (LSD, 5%). (Isinglass (IL1), isinglass (IS4), casein (CS4), potassium caseinate (CKS1), egg ovalbumin (AS1), gelatine (GL1), gelatine (GS2), gelatine (GS4).)

(Source: Cosme *et al.*, 2009)

The effect of this fining agent is due to the fact that a colloidal gel of molecules is added to the wine. This gel attaches electrically opposite charged particles and sometimes other non-charged particles present in wine become entrained in the complex, which then coagulates and precipitates sinking in (quite tight) flakes to the bottom. Influencing in this process is the pH of the wines, which should be low, because isinglass reacts better in acidic wines. The lower the phenol content and the alcohol content are, the better the fining will be (Jakob *et al.*, 1997 and Troost, 1988).
Although a high quantity of this substance is used in winemaking, only in France around 8.2 million hectolitres are fined with isinglass. It is exempt of declaration in Europe and overseas and there is no legal limit for this substance in wine. Fish allergy has been reported to affect 0.2%-2.2% of the European population. Fish can cause severe reactions in allergic individuals; therefore some assessments of isinglass in wine have been done and applied to EFSA. They concluded that the data submitted does not allow their Panel to consider the likelihood that isinglass used as fining agent in wine, will trigger an allergic adverse reaction in susceptible individuals, under the conditions of use stated by the applicant. The use of isinglass in wine is therefore allowed without need of declaration on the label (Weber *et al*., 2007; EFSA, 2007b).

3.6.4.5 Animal gelatine origin

Gelatine is a name derived from the Latin language *Proteinum ossii - gelatus,* meaning bones protein, stiff or frozen - and the first time this name was used regarding animal bone glue runs back to 1721 (Wahrig, 1972 in Troost, 1988). Gelatine is a protein that is prepared by mild hydrolysis of animal collagen-containing material, such the chief protein of skin, bones and connective tissues. It is thermally obtained from the acid, alkaline or enzymatic partial (almost complete) hydrolysis degradation of collagen (Troost, 1988; Ribéreau-Gayon *et al*., 2006 and Würdig and Woller, 1989).
Gelatine is part of scleroproteins, which plays the same role in the animal world as cellulose in the plant world. Gelatine has the task to act as a structural substance in the organism of animals and they are shaped like rods or wires. They are completely insoluble in cold water and in aqueous media. By prolonged heating in water it is generated through this heat soluble gelatine, which solidifies on cooling to a firm jelly. Gelatines have different characteristics according to thermal burden, that is the molecule mass diverges given to its diverse chemical and physical properties (Würdig and Woller, 1989).
A criterion for distinguishing different types of gelatine is the gel strength or jellifying power. This strength can be measure by a method used by an American named Bloom. The result is expressed in *Bloom grades* with values that are usually between 30 and 300 Bloom. High-Bloom gelatines have a bloom number of about 200-280 while low-Bloom gelatine has a number 50-100 (Troost, 1988; Würdig and Woller, 1989 and Ribéreau-Gayon *et al*., 2006).

Gelatine has different isoelectric points depending on its origin, pork or bovine and also depending on the type. Type A is formed by the acid processing of collagenous raw materials and exhibits an isoelectric point between 7 and 9. Type B gelatine is created by the alkaline or lime processing of collagenous raw materials and exhibits an isoelectric point between 4.6 and 5.2. This is above wine pH and therefore it is positively charged. Its molecular weight is around 15,000-150,000 and it contains about 85% proteins and up to 2% mineral residue (Burrough, 1996 and Margalit, 2004). Troost (1988) indicates that for oenological matters gelatine of type A with a low-Bloom value (60-100) should be used, as they are more efficient. It can be found in dry powder form or as liquid gelatine. The necessary swelling time influences the fining of the wine; therefore it is indicated to swell it at least for 5 hours. The quantity to be applied is from 5-30 g/hL. Gelatine can easily be subject of over/under-fining, therefore a pre-laboratorial test with test dosage between 2-20 g/hL should be considered. Tests must be carried out in order to determine the right quantity before it is applied. This test is important because the level of phenols and pH is really variable from wine to wine, so that gelatine will act divergent in each wine. A sensory evaluation is therefore also very important. Gelatine can be used alone or together with tannin or silica sol, usually in the ratio 1:10 (Troost, 1988 and Margalit, 2004).

Many authors reported the efficiency of gelatine as a wine fining agent and concluded that decrease of polyphenols depends on gelatine type. They likewise demonstrated that the method of addition has also a central meaning for the fining effect. To finalise they added that the temperature is a very significant factor. In temperatures over 20-25°C a clarification of the wine can no longer be reached. The lower the temperature the better, because a lower amount of agent is necessary and the lees are firmer, thus better filterability can be achieved (Troost, 1988; Rankine, 2004).

Gelatine uses, are as many other fining agents, not something new. It is often and almost only used for red wines. When applied it reduces astringency, some bitterness, and polymeric anthocyanins in red wines. It attacks mainly tannins and anthocyanins, therefore it is not only a clarifying agent but also has a massive influence on the taste and colour of the wine. It is useful in reducing the harshness of pressed wine. Gelatine interacts mainly with polymeric and monomeric phenolic compounds, as it can be seen in Figure 12 and in Figure 13. If used for white wines, which is not a common practice, it might be applied to clear cloudy wines that are hard to be cleared with bentonite. It is also helpful in reducing the bitterness after-taste in white wines (Troost, 1988; Margalit, 2004 and Cosme, 2009).

There is no legal limit for gelatine usage in wine, neither restriction concerning allergies. But there are defined compositions and charges by the oenological codex (Ribéreau-Gayon *et al*., 2006).

3.6.4.6 Vegetal origin

Different to other fining agents vegetal proteins are something much more recent in the wine industry, nevertheless there is already some literature that can be found (Christmann and Freund, 2004 and Neser, 2009).

Some authors mention the use vegetal proteins fining agent as a substitute for animal agents (Ribéreau-Gayon, 2006). There are some studies presenting the properties of some vegetal proteins, such as wheat gluten, soya, lupines, maize, pea or potato proteins in comparison with more commonly used fining agents. The most part of the studies report good clarification results (Marchal *et al.*, 2000 and 2002 Fischerleitner *et al*., 2002; Tschiersch *et al.*, 2010 and Gambuti *et al.* 2011).

In a study where they compared gelatine to rice protein, lupine protein, wheat protein, soya protein and pea protein, the plant proteins showed good clarification efficacy in red wines, where the effects were similar to gelatines. Except pea protein and lupine protein showed increase in wine turbidity, this didn't result in good clarification. The same study using above mentioned proteins have been done with white wine. The results conclusion of that study was that an adjuvant product (such as SiO2 or tannins) is necessary to achieve clarification. Furthermore they mentioned that if no adjuvant is added, protein elimination does not occur and the wine could be instable in what concerns protein (Mira *et al.*, 2006).

Pea protein

A pea protein consists of pure isolated vegetable-protein, which is produced without the use of chemical solvents from pea - *Pisum sativum*, commonly with a protein content of over 80%. The product is odourless and tasteless. It can be used in combination with silica sol for clarification, or as a single treatment indicated for reduction of phenols. The recommended dosage is 20 g/hL. It should be dissolved in water (in a tenfold volume) and while added to wine it should be constantly stirred.
Neser shows on her studies, comparing already used animal-based fining agents with pea proteins, that they have similar and comparable effect as fining agents (Neser, 2009).

The German company Erbsloeh has a product called *FloraClair,* which is claimed to be used in must or young wine for different means, clarification, flotation, and light tannin correction and for reduction of yellowish/brownish colorations (Erbslöh). Some advantages are that it does not need labelling as a potential allergen. The wine can be marketed as suitable for vegetarians and vegans and it is a non-GMO pea protein.

Cosme et al. (2011) recently researched the possibility of using non-allergenic pea protein or PVPP as alternatives for wine fining. She tested the effects these fining agents comparatively on phenolic compounds, browning potential, turbidity and sensory attributes. Globally the study comes to good and significant results; she achieved similar results for flavonoid and non-flavonoid phenols and wine colour where all were diminished. Potassium caseinate continues to be the most effective to combat browning, whereas for clarity pea-proteins work together with potassium caseinate. On sensorial analysis no difference ($p>0.05$) was found among wine samples set with different fining agents (Cosme *et al*., 2011).

Potato protein

Potato protein is a protein isolated from potato - *Solanum tuberosum*. This product is still in the development stage and is already currently commercially available in stores.

A commercial fining agent named *Vegecoll®* from the company Laffort, made from potato protein is presented by the company as being highly reactive in wines due to its elevated concentration of proteins and its zeta potential. Some advantages are that it does not need labelling as a potential allergen. The wine can be marketed as suitable for vegetarians and vegans; may be a substitute for gelatine (Iturmendi, 2013).

A request was submitted to the OIV to adjust the current legislation regarding the use of plant proteins in winemaking. The OIV OENO 28/2004 resolution was in conclusion amended in June 2013 to comprise patatin in the list of acceptable plant proteins (OIV OENO 495/2013) in wine. The European Union wine legislation is estimated to include patatin in 2014 (Iturmendi, 2013).

The protein content of the pulverized sample is around 90%. Use recommendations are the same as for pea protein fining. Patatin P is the name of a family of glycoproteins that can be recovered from potato aqueous by-product (Gambuti *et al.,* 2012).

Gambuti *et al.* (2011) reported results on patatin and say it is an apt alternative to animal proteins, gelatine, egg ovalbumin and casein, used as fining agent since it decreases the wine total phenols and tannins after the treatments with 10, 20 and 30 g/hL of commercial preparation containing Patatin. The efficiency in decreasing proteins reactive to wine polyphenols was patatin = gelatine > egg ovalbumin > casein ($p < 0.05$).

3.6.4.7 Metatartaric acid (E353)

Metatartaric acid is a polymerized tartaric acid that prevents potassium bitartrate dissolved in wine from forming crystals. Metatartaric acid may be classified as a dispersed polymer, because it is a mixture of polymers with different molecular weights. It is gained by heated tartaric acid, which passes through an inter-molar esterification, while water is released by the loss of acidity. There are many metatartaric acid preparations with different status of esterification. The lawfully imposed minimum rate is of 40%. A grade less than 30% would not be efficient on preventing crystal precipitation (Ribéreau-Gayon, 2006 and Mueller, 1976).

Grapes have both tartaric acid and potassium. Potassium reacts with tartaric acid in the finished wine resulting in potassium bitartrate. Depending on wine saturation of this salt, potassium bitartrate may precipitate out over some time as a clear, tasteless, odourless and crystalline material sometimes called “tartrate crystals” or “wine diamonds.” Tartrate crystals often precipitate out of the wine after bottling. Saturation is very temperature dependent (Jacob, 1991, Rankine, 2004; Christmann, 2008 and Troost, 1988).

Wines can reach its tartaric stability naturally or if the wine temperature is dropped to just above freezing for several weeks, but it may cost a lot of time and a lot of energy, respectively. If tartaric stabilization is not undertaken in wines that have a need for it, the wine crystals will appear in the bottle after bottling, especially if it is chilled before serving. It is not desirable to have this sediment in the bottle to be commercialised. Adding metatartaric acid to finished wine can inhibit the formation of wine crystals for some months depending on the temperature that the wine is stored as it can be seen on Table 12 (Mueller, 1976 and Scholten, 2003).

Metatartaric acid acts by conflicting the growth of the sub-microscopic nuclei around which crystals are moulded. It works by blocking the crystal growth. If the dose is too low, inhibition is only limited, and alterations and patchiness are detected in the shape of the crystals (Ribéreau-Gayon *et al.*, 2006).

Table 12 Relation between temperature and protection of metatartaric acid

Temperature in Celsius grades (°C)	Duration of protection
10	some years
10-12	> two years
10-16	at least 18 months
12-18	around one year
20	three months
25	one month
30	one week
35-40	Some hours

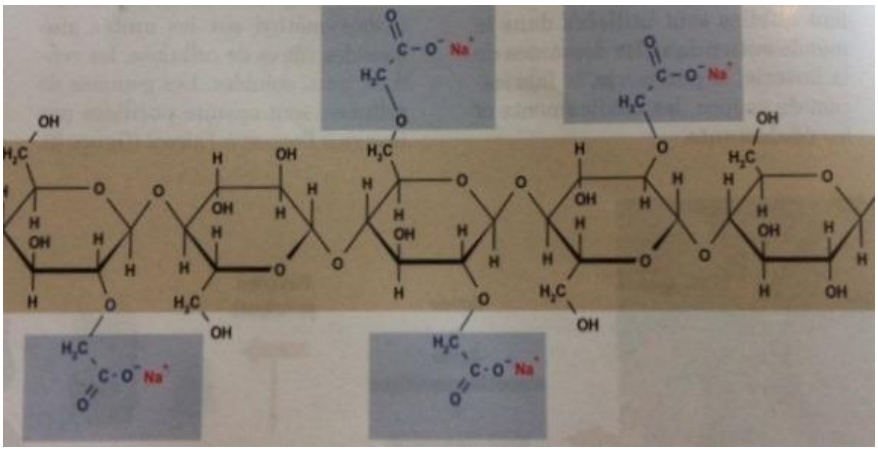
(Source: modified from Mueller, 1976 and Scholten, 2003)

By conductivity measurements Würdig *et al.* (1982) shown that not only the metatartaric acid inhibits crystallization, but also the dissolution of tartaric crystals in wine. Both effects can be used to determine metatartaric acid in the wine and up to a threshold concentration of 0.5 mg/l, in favourable cases even up to 0.2 mg/l.
Metatartaric use in wine is to avoid tartrate precipitation (potassium bitartrate and calcium tartrate), may be used legally in wine at quantities up to a highest of 10 g/hl (Ribéreau-Gayon, 2006).

3.6.4.8 Carboxymethyl cellulose (CMC)

Carboxymethyl cellulose, also called cellulose gum or sodium salt of carboxymethyl ether is a polysaccharide that derivate from cellulose. It is obtained by etherification of primary alcohol functions of the glucopyranose units. The functional properties of CMC depend on the degree of substitution of the cellulose structure. A CMC is, therefore, characterised partly by the degree of substitution (DS), and partly by its degree of polymerisation (DP), this can be seen on Figure 14 where DS is displayed in blue colour while DP is shown in brown. The blue shows the carboxymethyl groups and the brown colour shows the linear chain of cellulose. Like metatartaric acid, a polymer with a dispersed molecular weight, it has protective colloid properties in wine. For oenological use it is prepared exclusively from wood by treatment with alkali and monochloroacetic acid or its sodium salt (Ribéreau-Gayon *et al.*, 2006 and Resolution Oeno-366, 2009).
CMS MW ranges from 17,000 to 300,000 (degree of polymerisation from 80 to 1,500). The molecular weight can be evaluated through measurement of viscosity (Resolution Oeno-366, 2009).

Figure 14 Carboxymethyl cellulose (CMC) fragment



(Source: Brunner *et al*, 2013)

CMCs are available in the form of powder or white granules. The indicated dosage is around 4 g/hl. CMC solubility in water is variable (Ribéreau-Gayon *et al.*, 2006). CMC

acts in wine by covering the surface, and thus also the free valences of wine growing crystals (KHT only). Therefore further growth is blocked and depending on the size of the crystal, it is held in suspension and precipitation is prevented. CMC does not protect against Calcium tartrate precipitation (Missikiewitsch, 2010).
Recent studies by Brunner *et al*. (2013) says that if compared with other methods of tartaric stabilisation, CMC has the advantages of having a better environmental balance, which means less CO2 emission if compared with cold stabilisation and less water consumption in relation to electrodialise. Furthermore the study says it has a better efficiency, quality and less costs.

According to Codex Alimentarius it is regarded as a safe product and it is considered allergen-free and non-toxic (about 30 g/d). The maximal dosage is 10 g/hl (Resolution Oeno-366, 2009).

3.6.4.9 Compounds – mixture of different fining agents

Commercial products available as mixture are common in the wine industry. They have two or more fining agents together from organic, mineral or synthetic origin.
Although there are no restrictions or law against the use of vegetal proteins, as no necessity of declaration on the label, there are several cases of allergy reported to potato and to pea proteins (Monti *et al.*, 2011; Swert *et al.*, 2007 and Szymkiewicz, 2008).

3.6.5 Fining agents of inorganic, mineral and synthetic origin

3.6.5.1 Silica sol (Kieselsol)

A stable dispersion of solid colloidal silica particles in a liquid is called silica sol. This aqueous colloidal suspension of amorphous silica (SiO2) can be found in many different forms. The particle size varies in the range of 5-75 nm. They also differ from type to type of silica sol (Troost, 1988). Colloidal silica can be produced by neutralization with acid, ion exchange and from dialysis liquid sodium silicates (Won Kyun *et al*., 2009)
The form used in oenology is a white milky, cloudy, opalescent liquid, which can be found in different concentrations. The quantity to be used is dependent of the fining goal, quantity of combination agent and silica sol concentration. Some authors indicate 5 times of silica sol (in mL) of the gelatine concentration (in grams) e.g. 50ml/hL for 10gr/hL of gelatine (Troost, 1988 and Margalit, 2004).
Silica sol was acknowledged in the mid-20th century as a substitute for tannins for the fining of wine. In oenology it is mainly used for removal of other fining agents such as bentonite, activated carbon or copper sulphate. Usually used in combination with

gelatine or other proteins - egg white, isinglass, casein - (Troost, 1988). Silica sol is also known as taste-improving (Margalit, 2004). Troost (1988) showed the efficiency of silica sol on reducing bitterness of some wines.

3.6.5.2 Bentonite

Bentonite is hydrated aluminium-silicate clay that can be used to stabilize wine against the precipitation of soluble grape proteins, which can occur in wine when it passes through temperature increase. It is also known to reduce histamine from wine (Ribéreau-Gayon *et al.*, 2006).

Bentonites contain diverse exchangeable cations (Mg^{2+}, Ca^{2+}, Na^{2+}), which are dependent on geological origin. These cations play a major role in their physicochemical properties. Bentonite is basically an impure hydrated aluminium silicate, which when dispersed in water exists as exceedingly small flat plates or sheets. For winemaking purposes there are basically two types of bentonite, calcium and sodium. Sodium bentonite has flakes more widely spaced (100 Å) than those of calcium bentonite (10 Å). This difference in size reflects on the quantity of protein adsorption; calcium bentonite swells more in wine and has higher adsorption. Sodium bentonite adsorbs nearly five times its volume of water and at full saturation occupies a volume of twelve to fifteen times its dry weight (Rankine, 2004, Troost, 1988 and Ribéreau-Gayon, 2006).

The essential important factor of bentonite is its ability to adsorb proteins in grape juice and wine, and it is this especial primary characteristic that makes it so important in winemaking. This adsorption results from either an electrostatic attraction between positively charged proteins or the negatively charged bentonite (at wine pH). It can also be from adsorption of the protein molecules by hydrogen bounding. The charge on the protein molecule depends on the pH of the juice or wine and is usually positive, the lower the pH, the more positive the charge. Bentonite is not a selective absorber and may reduce other positive compounds of wine. Therefore the quantity of bentonite to be used should be as low as possible (Schmitt, 2012). Bentonite is also well known to reduce biogenic amines from wine. Schneider (2011) shows that if concentration of amines is high a dose of 400 g/hL can reduce until 70% of these biogenic amines.

The correct time to use bentonite is different concerning the type of wine. For white before bottling, following evaluation of their protein stability and red wine at the same time as fining. The factors affecting protein removal are making the correct choice for bentonite to be used, the amount added (it can absorb part of the aromas of the wine), the method of preparing the bentonite suspension, the acidity of the wine, the wine composition and it is of the most utter importance that the water used should be as pure as possible. The quantity to be used depends on the aim and on the producers' recommendation, but the effect of bentonite is negligible up to 40 g/hL. A suspension

should be prepared in water (5-15%) and left to swell. This suspension is put into wine. As for the quality of clarification, a temperature of 20°C is ideal, a bit higher than optimal temperature for other fining agents. After sedimentation and flocculation, wine can be racked (Rankine and Emerson, 1963; Rankine, 2004; Troost, 1988 and Ribéreau-Gayon, 2006).

3.6.5.3 Polyvinylpolypyrrolidone (PVPP)

Polyvinylpolypyrrolidone is a synthetic material, which has a higher molecular weight than polymer of polyvinylpyrrolidone (PVP).

It is difficult to determine the exact MW of PVPP due to its lack of solubility in common solvents. PVPP is well known in oenology for its strong affinity to phenols (Margalit, 2004 and Ribéreau-Gayon *et al.*, 2006).

PVPP is a white powder, sold as "Polyclar", it has been used since the middle of the 20^{th} century to stabilise diverse beverages, including wine and being the first synthetic wine fining agent. The quantity to be applied is dependent on fining aims and on the amount of phenolics to be removed. Indications are between 10 and 40 g/hL and legally it has a maximum dosage of 80 g/hL in countries belonging to the EU and another number of countries overseas (Rankine, 2004 and Ribéreau-Gayon *et al.*, 2006).

The method of application of PVPP in wine is to mix the appropriate amount determined by a trial. With continual stirring to the bulk wine and maintaining the mixing long enough to ensure good contact between the insoluble fining and the wine. There is the possibility to incorporate PVPP into filter sheets, which enable the wine to be treated continuously (Rankine, 2004).

In oenology PVPP uses is for minimizing a tendency to browning the wines, particularly in pressings, which also leads to astringency. It is also used to remove or prevent pinking, by absorbing the precursors of those pigments. It is used to stabilise blush or rosé wines. PVPP is considered to be more adsorptive to phenols without decreasing the aromas of wine. It is used for colour reduction on white wines with combination of carbon or casein. The last combination is also used to reduce wine maderisation. Finally PVPP is applied for reduction of bitterness in white and red wines (Rankine, 2004; Ribéreau-Gayon *et al.*, 2006; Jakob *et al.*, 1997; Margalit, 2004; Christmann, 2001; Würdig and Woller, 1989 and Troost, 1988).

As PVPP is a polyamide it interacts with phenolic compounds such as other agents. PVPP binds polyphenolics by hydrogen bonding between the PVPP-carbonyl group with the phenolic-hydroxyl groups of both simple phenolics and flavonoids (Donel *et al.*, 1993 and Cosmo *et. al.*, 2011).

PVPP eliminate tannins, oxidisable cinnamic acids and quinones formed when they oxidise. PVPP reacts specifically with low polyphenols such as monomers and dimers, e.g. catechin and anthocyanin. The binding action on leucoanthocyanins, catechins,

flavonols and phenolic acids, as mentioned above, take place between PVPP carbonyl and the phenolic hydroxyl. As the PVPP is insoluble, the phenolic molecule adsorbs on its surface and precipitates out of the solution (Rankine, 2004; Ribéreau-Gayon *et al.*, 2006; Jakob *et al.*, 1997; Margalit, 2004; Würdig and Woller, 1989 and Troost, 1988).

3.7 Scope of Work

This work attempts to study techniques and methods of reducing the allergic potential arising from animal based fining agents, specifically from egg and milk. The chosen methods of reduction are through the utilisation of different filtrations and separations after fining the wine. The analytical control of the fined and then treated wines is done through immunological detection as well as clinical validation, which without them the EFSA do not consider as complete research results in the allergic-analytical area. The research was conducted in four parts:

1. The first part was an attempt to measure the residues of fining agents in wine. Furthermore to produce fined wines with no or very low fining material residues by applying filters or separation methods commonly used by the wine industry.
    a. The main objective of this part is to quantify the effect of filtration process on the residues of fining agents
    b. This part have been conducted in many different trials during three research years;
        - In the first year the intention was to create a worst case scenario
        - In the second year the intention was to approach the research to wineries common practices and reality
        - In the third year the intentions was to repeat some of the trials in an optimised way

2. The second part of the project was to analyse the wine residues through ELISA
    a. The first phase involved the development of specific ELISA to the matrix wine; white and red

3. The third part of the project investigated in humans the risks these residues may trigger
    a. The first phase involved recruiting allergic subjects all over Germany
    b. The second phase involved medical allergic tests in patients with allergy to egg and milk

4. The forth part of the project is to assess the wine quality after all process
    a. Though sensorial analysis

b. Reporting how this research may be applied on the GMP of wineries is also included on the intentions of this work.

Figure 15 Scope of work - project layout

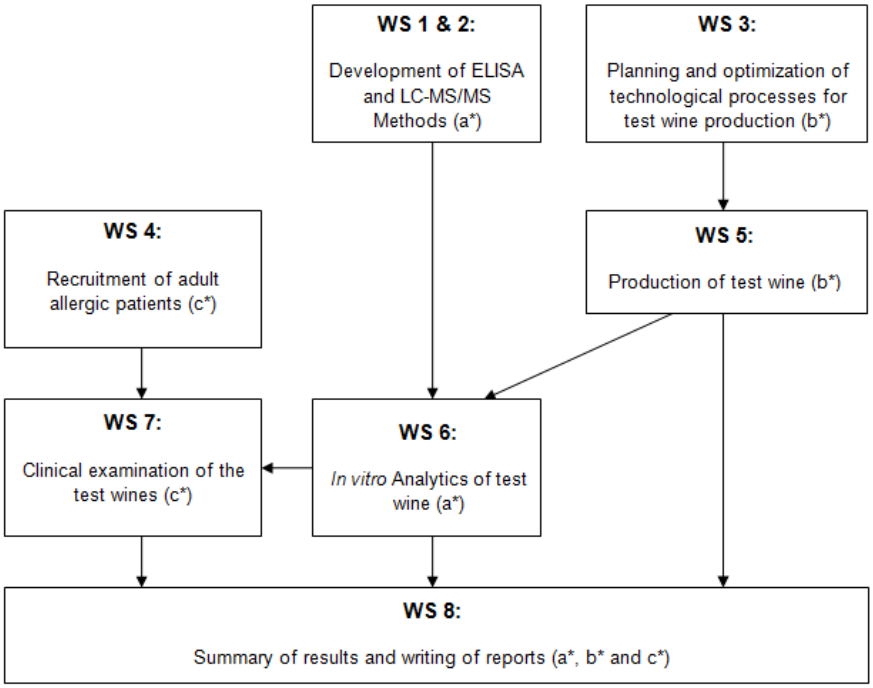


Diagram 1: detailed description of the proposed solution path and working steps (WS) *a: University of Hamburg, b*: Geisenheim Research Center, c*: Munich TU.

3.7.1 Project (FEI)

The studies for this doctoral thesis are based on an official project; number AiF 16330 N, which was funded under the program to promote Industrial Joint Research (IGF) of the Federal Ministry of Economics and Technology (via AiF) by the research Association of the German Food Industry (FEI).

This project took place in three different institutions in a partnership, which is a cooperation between the Research Centre of Geisenheim – Department of Oenology and Wine Technology, the University of Hamburg - Institute of Food Chemistry and Munich Technical University - Department of Dermatology and Allergology. These institutions are respectively responsible for: cellar technologies - filtrations, alternative fining agents, and all involving wine production; developing ELISA for matrix white and red wine and HPLC-MS analysis of white wine; and clinical & medical investigations with humans.

4 Material and Methods

This study was carried out with the intention of determining by accurate methods of detection the existence of residues arising from fining agents used during the wine making process. The possibility of detecting the fining agent protein by LC MS/MS-methods was raised by a project partner, but due to better precision and detection sensitivity the ELISA method was chosen.

The study is based on specific proteins that come from chicken egg albumen and from milk, i.e. ovalbumin, lysozyme and casein. This study also had the intention of testing whether other oenological methods were reasonable alternatives, in order to provide better approaches to reducing residues, as it is now officially required to declare any residue over 0.25 ppm on the label.

Throughout the course of this study, and influenced by its collaboration, major changes in labelling law happened. By means of approved analytical methods recommended by the OIV, it can be considered that casein, ovalbumin and lysozyme presence in the final wine for consumption is 'detected' just when the analytical values acquired are over than the detection limit value set at 0.25 mg/L. Only in these 'detected' cases is labelling obligatory. If these methods do not identify any protein from the food allergen in the wine, at that point it could be considered that no residue beyond the detection limit is present. In this way the industry could avoid overuse of precautionary labelling placing severe restrictions on dietary choices for consumers.

4.1 Trials

4.1.1 First trial – "worst-case scenario"

In 2010 two wines; a white and a red (wine No. 1 and 2 from results chapter), and two different concentrations of fining material was used for the first trial. The first concentration "A" was based on wine law, if existent or on recommended dosage by the fining agent producer. The second dosage "B" was the double of the first used concentration (B= 2xA), to create a worst-case scenario, in which the study is also able to quantify the residues even in a case of over dosage.

A further intention of this study was to check the difference in quantity of residues between a "normal dosage" "A" and a worst-case scenario "B". This trial can be seen on Diagram 3 and can be found in a detailed diagram in the annexes chapter. Apart from ovalbumin and casein that are listed as allergens by the EU food law, further

fining agents were used as alternatives, since this study also considered the sensory effects of used fining agents on wine.

Table 13 List of fining agents used on the first trial

Primary matter	Protein/material	Concentration (A - B) g/hl	Commercial name	Producer
Cow milk	Casein	40 – 80 (400-800 ppm)	*Kal-Casin Leicht löslich®*	Erbsloeh[+]
	Whey-protein	44 – 88 (440-880 ppm)	No commercial name	Erbsloeh
	Potassium caseinate (PVPP)*	50 - 100 (500-1000 ppm)	*SensoVin®*	Erbsloeh
Chicken eggs	Lysozyme	50 – 100 (500-1000 ppm)	*SihazymLyso®*	Begerow
	Egg-white protein	16 – 32 (160-320 ppm)	*AlbuVin®*	Erbsloeh
Plants	Pea-protein	30 – 60 (300-600 ppm)	*FLoraClair®*	Erbsloeh
	Potato-protein	30 – 60 (300-600 ppm)	No commercial name	Begerow
Pork	Gelatin	10 – 20 (100-200 ppm)	*ErbiGel®*	Erbsloeh
Fish	Isinglass	6% suspension - double	*Hausengranulat Drifine®*	Erbsloeh
Synthetic	PVPP	60 – 120 (600-1200 ppm)	*Polyclar®*	Erbsloeh

*modified PVPP, two different adsorbing silicates.[+]Erbsloeh: all technical product leaflet may be found under: http://www.erbsloeh.com/en/products/wine/products_a_z#product_B

Figure 16 Picture of fining agents

The process of fining has been carried out in big balloon glasses of 110 litres. To avoid cross-contamination the same balloon has been used for only one sort of fining agent; therefore resulting in a number of 10 balloons, one for each of the 10 fining agents plus the one used for control; non-fined wine. Fining agents have been weighted in the laboratory and dissolved as indicated by producer and stirred with magnetic fish until it was completely dissolved.

Figure 17 Glass balloons in the cellar

(Source: private pictures)

Treatments after wine fining

Wines were fined with both different doses as presented in Table 13. After 24 hours further treatments took place. These can be seen in **Fehler! Verweisquelle konnte nicht gefunden werden.**. They include different filtrations, separation, heat and finings - here used as reduction strategies.

Table 14 After fining treatments

Treatment	Properties	Commercial name	Company
Filtration	filter pads - ⌀ 0,45µm filter pads - ⌀ 1-3µm	"*SEITZ EK*" and K100"Cellulose (size 200x200 mm)	Pall Corportion
	membrane cartridge -⌀ 0,45µm	"*SEITZ-MEMBRAcart*" type 419A, grade B (Beverage version)	Pall Corporation
	cross-flow - one module – defined within microfiltration rates	*"Sartflow Compact"* with a pump: 15-20 m^3/h against 2.5 bars	Sartorius
	diatomaceous earth -fine kieselgur	Fine-Kieselgur	Pall Corporation
Separation	centrifuge – 7500 rpm (1500 l/h against 3,5 bar)	"*Tellerseparator SB14*" 7500 rpm at 1500 L/h against a pressure of 3.5 bars.	Westphalia
Heat	flash-pasteurisation – 20s at 72°C	Plate heat exchanger	FAG
Fining	bentonite – Mix (Na^+Ca^{2+}) 200g/hl (12 hours in water)	*"Aktivit®"*	Erbslöh
	silica sol - (SiO_2) 50ml/hl	*"Klar-Sol 30®"*	Erbslöh

Figure 18 Filters in the FAG cellar

(Source: private pictures)

Samples of wine have been taken before and after every procedure and treatment and have been sent to Hamburg to be analysed by ELISA tests. Comprehensive sensorial analyses have been carried out with all 320 samples (only “sample 3” or “sterile filtered wines “of Figure 19).

On Figure 19 “Non-filtered” wine, is the control wine. “Fined wine” is the wine after fining. “Treated wine“ is the wine after a filtration, separation or any treatment done to reduce the fining agents from wine and finally “Sterile filtered wine” is the wine with a final sterile filtration.

Figure 19 Flow set out of first trial

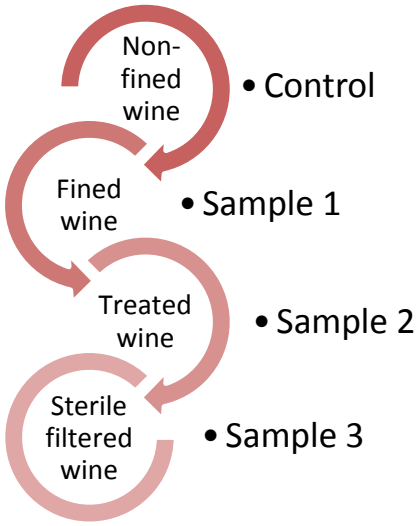
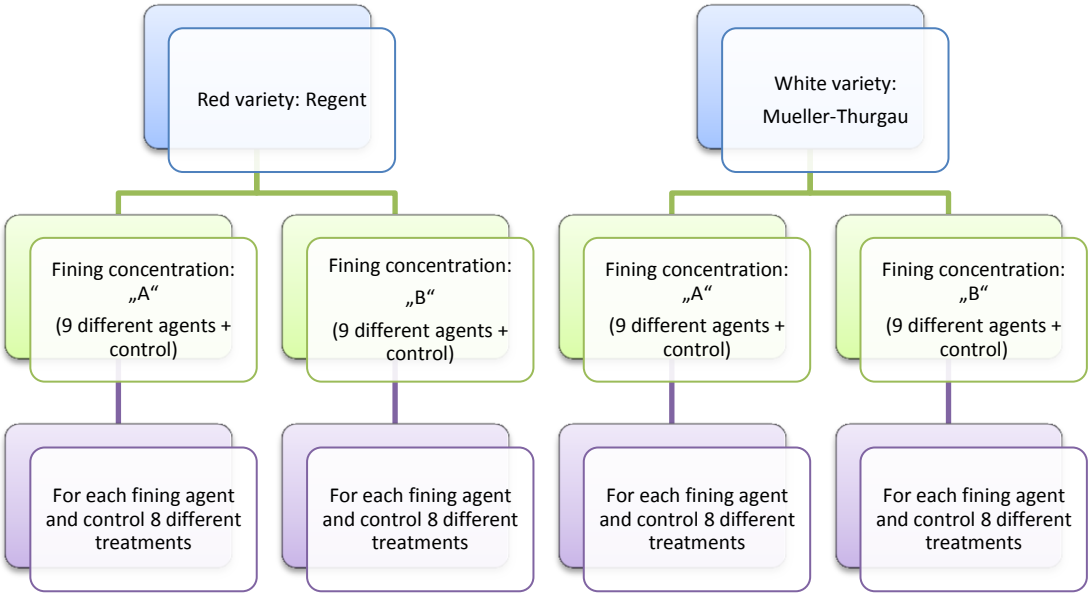


Diagram 3 First trial diagram



A more comprehensive diagram of this trial is displayed on annexes chapter.

4.1.2 Second trial – “normal quantities applied in common wineries”

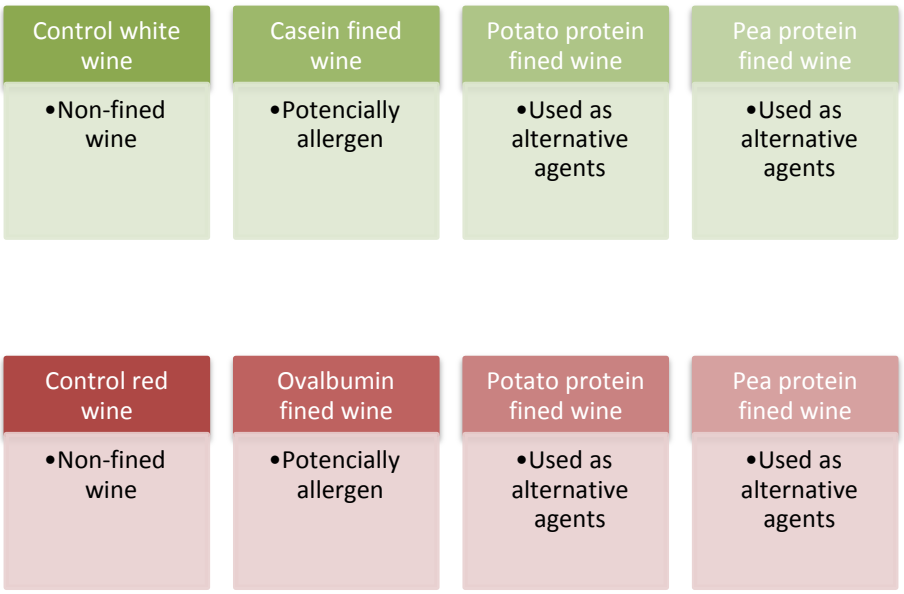
For this trial done in 2011, the appropriate dosage amounts of the fining agent have been determined by preliminary analytical and sensorial tests of the base wines, as commonly done at wineries. This trial is divided in two parts and it is a partial repetition of the first year together with an attempt to achieve better results. Furthermore results closer to reality on the sensory analysis in the first part and to improve previous trial of lysozyme on the second part of this trial.

4.1.2.1 First part – alternative fining agents

In this trial white wines have been fined with casein, here set as the allergic potential protein whereas for red wines egg protein has been used instead of casein. All white and red wines (Wines No. 3 to 8 from results chapter) have been fined with potato and pea protein, here used as alternative fining agents to compare its effect against casein, in white wines and egg proteins in red wines. The comparison was tested through sensorial tests and the residues through ELISA, in Hamburg University - project partner. To estimate the required fining agent dosage amounts, the wines were fined in the preliminary tests with 5, 15 and 20 g/hL in 500mL glass cylinders. After an exposure time of 24 hours, the samples were examined analytically on turbidity, phenol content and protein stability.

For measuring of the samples turbidity they were thoroughly mixed and measured by means of NTU (Nephelometric turbidity value). For determination of the clarifying effect, the samples were centrifuged and the turbidity content measured once more. Finally, it was sensorial tasted by a small panel, which aimed to choose an optimum of fining agent amount - mainly based on wine astringency together with phenolic content. Upon the results obtained from the preliminary test the dosages for the main experiment have been defined. The same concentrations of the diverse fining agents have been set for the same wine variety. The aim here is the possibility of tasting comparison later on. Concentrations used can be found on Table 15.

Figure 20 Second trial white and red wines schema



4.1.2.2 Second part – lysozyme and metatartaric acid

This trial is based on lysozyme treatments together with metatartaric acid. Only one red wine – Sangiovese and one white wine – Riesling has been used here (wines No. 9 and 10 from results chapter). Each wine was separated in 4 glass balloons and weighted up to exact 25 L. The first balloon was the control (sample K), the second balloon was only treated with lysozyme (sample L), while balloon 3 have been treated with lysozyme and fined with bentonite (sample LB) and balloon 4 was treated as follows:

- Wine was treated with lysozyme
- After 24 hours it was fined with bentonite
- After further 24 metatartaric acid was added

All samples have been sterile filtered and bottled in 0.75 L bottles. These bottles have been kept in the FAG-cellar with "cellar-temperature" around 17°C for three weeks. But two groups of sample LBM (samples with metatartaric acid - which is sensitive to temperature) have been kept in different temperatures for the same three weeks. The first groups was kept by constant 30°C (T1) and the second group by alternate temperature between 17°C and 30°C, changing it every three days. Temperature changes have been simulated by a heating cabinet where temperature can be programmed. The intention of this change in temperature is to understand if after metatartaric acid breaks down, due to high temperature, the content of lysozyme is changed or remains the same.

Figure 21 Second trial white and red wines schema for lysozyme

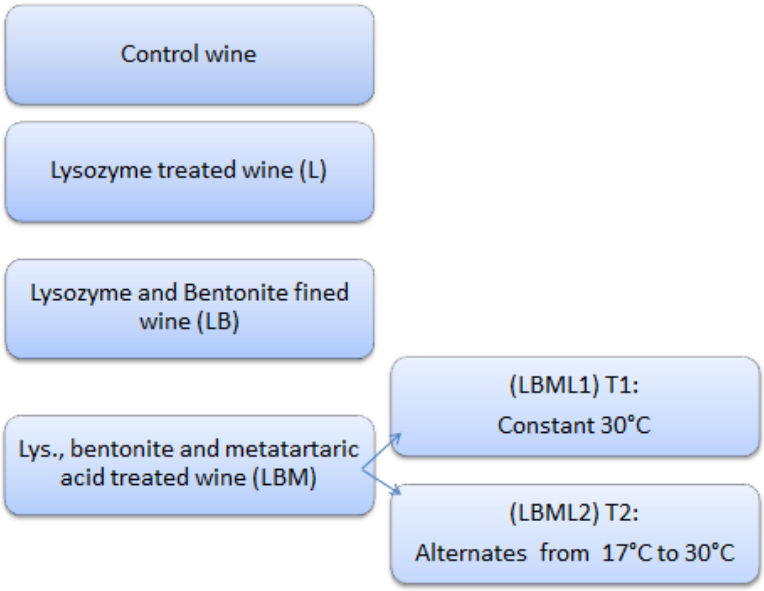


Table 15 List of fining agents used on the second trial

Wine	Protein/material	Commercial name	Concentration g/hl
White Cuvée*	Casein	*Kal-Casin Leicht löslich*	20
	Pea protein	*FloraClair*	
	Potato protein	Begerow	
Riesling	Casein	*Kal-Casin Leicht löslich*	10
	Pea protein	*FloraClair*	
	Potato protein	Begerow	
Mueller-Thurgau	Casein	*Kal-Casin Leicht löslich*	20
	Pea protein	*FloraClair*	
	Potato protein	Begerow	
Red Cuvée**	Ovalbumin	AlbuVin	5
	Pea protein	*FloraClair*	
	Potato protein	Begerow	
Pinot Noir	Ovalbumin	AlbuVin	5
	Pea protein	*FloraClair*	
	Potato protein	Begerow	
Sangiovese	Ovalbumin	AlbuVin	20
	Pea protein	*FloraClair*	
	Potato protein	Begerow	
Riesling	Lysozyme + Bentonite + Metatartaric acid	Begerow + Erbslöh +Erbslöh	50 + 200 + 10
Sangiovese	Lysozyme + Bentonite + Metatartaric acid	Begerow + Erbslöh+ Erbslöh	50 + 350 + 10

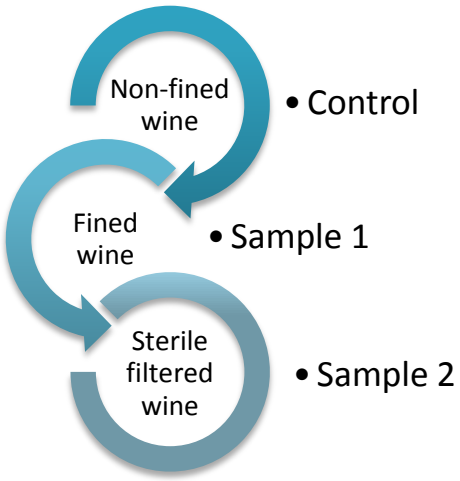
*(different German sp. *vinifera* varieties) ** (at least 50% of Pinot Noir mixed with other varieties)

A non-fined sample of each wine such as all wines showed on Table 15 have been settled, racked, EK/sterile filtered and bottled after the fining procedure. Fining has been done exactly as on trial 1; fined and settled for 24 hours, but in smaller glass balloons, this time of ≈25 litres. For the lysozyme samples, after the first 24 hours a second fining, now with bentonite (prepared by soaking it in water for 12 hours before being added to wine) took place and the wine have settled for other 24 hours, before being racked and treated with metatartaric acid and finally being filtered.

Figure 22 25L Glass balloon being filled up

Samples of wine have been taken before and after every procedure and treatment and have been sent to Hamburg to be analysed by ELISA tests. A sensorial analyse have been carried out with samples "sample 2" or "sterile filtered wines "of Figure 23.

Figure 23 Flow set out of second trial



4.1.3 Third trial - "repetitions + Carboxymethyl cellulose" (CMC)

On the third year; 2012, trials are again slightly different or improved from previous years. This time trials are divided in three parts.

4.1.3.1 First part – Lysozyme (third repetition)

This trial has been carried out in small scale, in the laboratories of the FAG with a more comprehensive number of wine varieties, than previous year: (3 whites and 3 reds – wines No. 11 to 16 from results chapter) and fining combinations as showed on Table

16 and Table 17. The treatments are similar to the one carried out on second trial – second part, but with one different fining agent; CMC, which has a similar activity and function as metatartaric acid in wine. Wines have been fined on 500 mL glass cylinders. The CMC used was from the company Erbsloeh.

Table 16 List of fining agents and its [] used on the third trial -first part

Agent	Abbreviation	[]g/hL	ppm
Lysozyme	Lyz	30	300
Metatartaric acid	Meta	10	100
Bentonite	Bento	400	4000
CMC	CMC	10	100

Table 17 Samples of third trial -first part

	Samples + fining
1	Control
2	Lysozyme
3	Meta
4	Lyz+Meta
5	Lyz+Bento+Meta
6	Lyz+CMC
7	Lyz+Bento

On this trial the residues of some fining deposits have been photographed on graduated cylinder, to observe and relate the fining agent deposit quantity. While others have been centrifuged and dried overnight and photographed on the microscope to study the crystal formation.

4.1.3.2 Second part - Casein and Ovalbumin (third repetition)

For this trial three white varieties of wine (see Table 18) have been fined with 10 g/hL of casein, furthermore the same tree varieties have been used as control. Likewise three red wines have been fined with the same quantity; 10 g/hL, but with ovalbumin agent. Fining agents used are the same as showed on Table 13 (Kal-Casin Leicht-löslich and AlbuVin). After 24 hours wines have been bottled without being filtered. This trial has the intention to check the residues of wines that are fined at normal dosage and are furthermore non-filtered, as some wineries works. The residues are checked by ELISA test in Hamburg University project partner.

4.1.3.3 Third part - Flash pasteurisation and centrifugation (second repetition)

This trial is just a repetition of the first trial – flash pasteurisation and centrifugation (**Fehler! Verweisquelle konnte nicht gefunden werden.**) since ELISA results were very high on the first year and therefore needed to be repeated and proved. For this trial a white wine Mueller-Thurgau have been fined with 40 g/hL of casein and a red cuvée wine (over 80% Pinot Noir) have been fined with 16 g/hL of ovalbumin, again the same as showed on Table 13 (Kal-Casin Leicht-löslich and AlbuVin). All procedures have been the same as on first trial. The residues are checked by ELISA test in Hamburg University project partner.

Table 18 List of wines used on the third trial

Wine variety		**Trial part in which the wine have been used**		
		First part	**Second part**	**Third part**
White varieties	Mueller-Thurgau	**√**	**√**	**√**
	Riesling	**√**	**√**	
	Chardonnay		**√**	
Rosé wine	Pinot Noir	**√**		
Red varieties	Cabernet Sauvignon + Merlot (Cuvée)	**√**	**√**	
	Dornfelder	**√**	**√**	
	Pinot Noir	**√**	**√**	
	Cuvée (over 80% Pinot Noir)			**√**

4.2 Analyses

Wine analyses were done shortly before every trial.

General wine analyses or analysis of conventional oenological parameters

The following analyses operations are defined according to Jacob *et al.* (1997) on chapter 11:

- Alcohol (calculated in refraction number)
- Sugar (*Rebelein*)
- Sugar-free Extract (calculated in refraction number)
- Relative density (density meter with oscillating U-tube installed)
- pH and Titratable acids (SET-Titrino)

Further analyses are described each one separately with more details:

- Colour measurements (CIELAB)

- Total phenols (Folin-Ciocalteu reagent – mg/L and HPLC)
- Anthocyanins mono and diglucoside (HPLC, cyanidin, delphinidin, malvidin, peonidin and petunidin – mg/L)
- Oxidative capacity (Trolox/L)
- Conductivity (µS)
- Minerals (TRFA - mg/kg)
- ELISA (direct and indirect sandwich - ppm)
- Sensory analysis (triangle, descriptive and ranking tests)

4.2.1 Colour measurements

Wine colour was analysed using the CIELAB-System (1976). The intention of this analysis was to observe if there were differences between fining agents.

Principle

The CIE-L*a*b* system is a three-dimensional chromatic colour space, which was established by the International Commission on Illumination, in French 'commission internationale de l'eclairage': CIE. This system that is a modification of one of the first mathematically defined colour spaces is based on the theory of opposite colours. As written at the CIEs electronic page by using the device independent-colour-3D-model it is possible to determine numerically the differences in colour. The model is objective and is designed to approximate human vision, or in terms of their colour and intensity, similar to describe how they are perceived by the human eye. (Valdes, M. *et al*., 1997)
In this way the three coordinates of CIELAB system - constituted by L* a* b*, is representing respectively, the lightness of colour (L* = 0 yields black and L* = 100 indicates diffuse white) the luminosity, red (a*)/green (-a*) tonalities and yellow (b*)/blue (-b*) tonalities. All colours are represented within a solid, in which the central axis L* varies between 0 and 100% (completely transparent and opaque). The asterisk (*) is used to indicate each coordinate, for this reason used after L, a and b (Bakker, J. *et al*., 1986). Both coordinates, a* and b* form a horizontal plan within this solid. It can recreate a colour similar to the real wine one colour.

Execution

The wine sample is measured with a spectrophotometer model Dr. Lange Cadas 200 – Spektralphotometer, in a disposable plastic cuvette (10 mm) at 380 to 780 nm absorption length. Thereof the blank value is abstracted. Data is worked in an Excel table that gives L*a*b* values; L* (lightness), a* (measure of redness), b* (measure of yellowness) Further Software programs, such as Adobe Photoshop, can generate visually the supposed real colour of the wine based on its measured three-dimensional - L*a*b* values. Another possibility to detect if a visual perceivable difference exists is by using following formula:

$$\Delta E = ((L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2)^{0.5}$$

If $\Delta E > 1$ there is a visual perceivable difference exists.

4.2.2 Phenols

The quantification of total phenols was determined with Folin-Ciocalteu reagent (FCR) method. Fining agents reaction partners are mainly phenol contents of wine, therefore this analysis was important to comparison between fining agents.

Principle

Phenolic compounds when in alkaline milieu with FCR result in a blue colour that can be measured photometrically at 720 nm. In doing so, the hetero-poly acids become shortened into a blend of +5-+6 significant bonds through phenolic bonds, which conducts to the formation of a bluish complex molybdenum-tungsten. These formed chromogens can be detected spectrophotometrically. Fructose in higher levels, ascorbic acid, iron (II) ions and sulphur dioxide disturb these reactions and mislead the results of the total phenols. Those substances should therefore be oxidized with H_2O_2 before the determination.

Folin-Ciocalteu reagent, a mixture of phosphotungstic (H3 PW12 O40) and phosphomolybdic (H3 PMo12 O40) is a clear, acidic solution with a bright yellow colour. It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent. The reagent will react with phenols and non-phenolic reducing substances to form chromogens that can be detected spectrophotometrically, as mentioned above.

The hetero-poly-acids are reduced to blue oxides during phenol oxidation. For a complete reaction of phenols to go through phenolation and then, oxidize to quinones, it is necessary to have an alkaline medium, which is established by the addition of sodium carbonate.

Execution and chemicals

- Folin-Ciocalteu-reagent (Merk, Darmstadt)
- Solution of carbonate of sodium Na_2CO_3 (200 g/L)
- Hydrogen peroxide H_2O_2 (30%)
- Catechin

The measurement is done using a spectrophotometer at 720 nm wavelength.

Sample preparations starts with elimination of SO_2 or ascorbic acid with H_2O_2, by mixing 50 mL sample with 0.2 mL 30% H_2O_2 solution and wait time of 30 minutes.

The prepared sample (1 mL) is added to a 100 mL volumetric flask and diluted with 75 mL of distilled water, plus the addition of 5 mL of Folin-Ciocalteu reagent and it is

further mixed and left for 3 minutes apart. Next step is the addition of 10 mL of sodium carbonate and the volume is filled to 100 mL in the flask with distilled water.
After one hour it must be measured in a cuvette (1 cm) at 720 nm absorption length. Thereof the blank value - normally water, is abstracted. The value is given in mg/L of (+)-catechins.
A standard curve, done with a buffered solution of catechin, helps to estimate the final concentration of equivalent in catechins (mg/L); the standard curve is linear with r^2: 0.9991 between 0 and 1000 mg/L.
The standard curve is a stock solution with 500 mg of catechin per litre, prepared by dissolving the corresponding substance in absolute alcohol (e.g. 50 mg catechin/100 mL alcohol). This solution is pipetted into 10 mL small volumetric flasks as follows: 1.0; 2.0; 4.0; 6.0; and 8.0 mL and the flasks are then completed to the right volume with absolute alcohol. These dilutions will contain respectively: 50; 100; 200; 300 and 400 mg of catechin.

4.2.3 Anthocyanins and non-coloured polyphenolics identification

The identification of anthocyanins and non-coloured phenolics was done using High Performance Liquid Chromatography (HPLC) using the Standard Operating Procedures (SOP-WG2) of the wine chemistry laboratory from the research centre of Geisenheim.

Non-coloured polyphenols

This analysis is also done using HPLC of a fluorinated RP-Phase with SOP-WG2-34

Principle

This method is good for the quantitative determination of individual polyphenols, i.e. it is suitable for both; the anthocyanins and the colourless phenols. Especially in the area of apolar polyphenols (flavonols, dihydrochalcones, etc.) with this method a good separation can be achieved (Klumpp, 2004). In the present thesis Fluofix column was only used for identification and quantification of colourless phenols.

Execution and chemicals

- O-Phosphoric acid (85%)
- Bi-distilled water
- Acetonitrile (HPLC grade)
- Methanol (HPLC grade)

HPLC solvents:

Flux material A: water + phosphoric acid (99.5/0.5) (v/v/v)

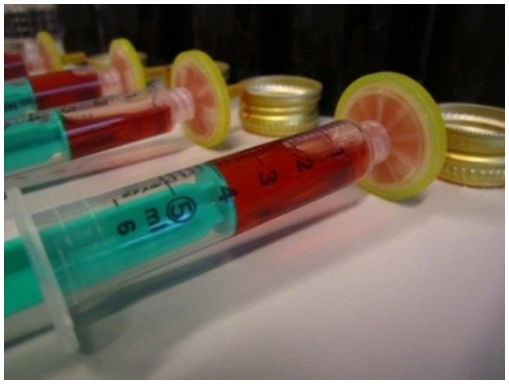
Flux material B: water + phosphoric acid + Acetonitrile (49.5/0.5/50) (v/v/v)

Flux material C: water + methanol (50/50) (v/v) used to clean the column.

Apparatus:	
Pump:	DIONEX LPG 3000 HPLC Pump
Detector:	Thermo Scientific Finngan Surveyor PDA Plus Detektor
Wave length:	280, 320, 360 nm
Column:	FLuofix 120 E, 2*125 mm, 5 µm with prior column
Column heater:	Thermo Scientific Finngan Surveyor Autosampler Plus
Data:	
Flow rate:	0.19 mL/min
Pressure:	around 40 bar
Temperature:	20 °C
Injection:	Thermo Scientific Finngan Surveyor Autosampler Plus
Injection volume:	2 µL
Time length one run:	around 45 minutes
Integration with PC by:	Chromeleon Client Program Version 6.8

The samples are filtered with 0.45 µm membrane and put into the sample vials.

Figure 24 Membrane-filter (0.45 µm) for HPLC samples



The calibration line is done using existing standards, for example, caffeic acid, chlorogenic acid, coumaric or ferulic acid would be calibrated, and derivatives of the acids are calculated as the corresponding acid.

Evaluation is done by:

- Identification of catechins and bounds of hydroxybenzoic acid (280 nm)
- Identification of hydroxycinnamic acid (320 nm)
- Identification of flavonol glycoside (360 nm)

Results are given in mg/L.

Anthocyanins (SOP-WG2-05)

Principle

Anthocyanins absorb light in the range 510–535 nm, for this reason it is easy to detect these pigments. In this study we used a detector (UV-Vis-Detektor) at 520nm. The anthocyanins sum count with 17 different forms of anthocyanins. For the variety Regent most of its anthocyanins are diglucoside, because it is a hybrid variety, but in this work the result is calculated specifically in mg/L of Malvidin-3-glucoside as described in the SOP-05 modified from Marx, R. (2000).

Anthocyanins measured in HPLC sub-dived and eluted in groups

Non-acylated

- Delphinidin-3,5 -di-glucoside
- Cyanidin-3,5 -di-glucoside
- Petunidin-3,5 -di-glucoside
- Delphinidin-3-glucoside
- Peonidin-3,5-di- glucoside
- Malvidin-3,5-di- glucoside
- Petunidin-3-glucoside
- Peonidin-3-glucoside
- Malvidin-3-glucoside

Acetylated

- Malvidin-3-acetyl-glucosid

Coumarylated

- Petunidin-3,5-coumaroyl-diglucoside
- Delphinidin-3-coumaroyl-glucoside
- Peonidin-3,5-coumaroyl-diglucoside
- Malvidin-3,5-coumaroyl-diglucoside
- Petunidin-3-coumaroyl-glucoside
- Peonidin-3-coumaroyl-glucoside
- Malvidin-3-coumaroyl-glucoside

Execution and chemicals

- O-Phosphoric acid (85%)
- Bi-distilled water
- Acetonitrile (HPLC grade)
- Methanol (HPLC grade)

HPLC solvents:

Flux material A: water + phosphoric acid + Acetonitrile (94/2/4) (v/v/v)

Flux material B: water + phosphoric acid + Acetonitrile (48/2/50) (v/v/v)

Flux material C: water + methanol (1/1) (v/v) used to clean the column.n un

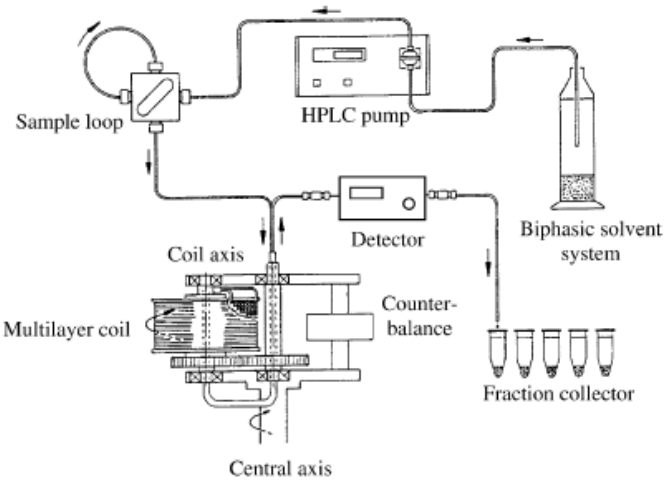
- Malvidin-3-o-glucosid (Oenin) for Standard

Apparatus:

Pump:	DIONEX P680 HPLC Pump
Detector:	DIONEX PDA-100 Photodiode Array Detector
Wave length:	200-650 nm (spectrum); Max. absorption 520 nm
Column:	LiChrospher 100 RP 18, 5 µm, 250*3 mm with prior column (Merk)
Column heater:	DIONEX STH 585
Data:	
Flow rate:	0.5 mL/min
Pressure:	around 80 bar
Temperature:	20 °C
Injection:	DIONEX ASI-100 automated sample injector
Injection volume:	20 µL
Time length one run:	around 65 minutes
Integration with PC by:	Chromeleon Client Program Version 6.4

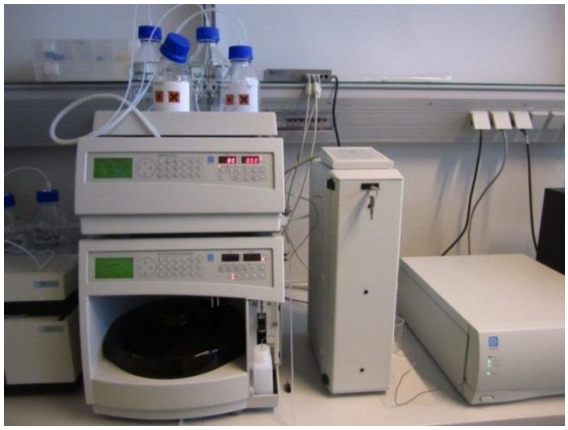
The execution of this analyse is done by filtering the sample with membrane filter of 0.45 µm into the sample vials and placing them in the apparatus.
The calibration line is done using a stock solution of Mal-3-o-glc in different concentrations as described in SOP-05.
The identification of the substances was performed by comparison of retention times and spectra with HPLC-DAD detector. The quantification was based on the relative peak area fractions of the total area of the anthocyanin peaks in the sample with incorporation of the calibration line.

Figure 25 Example of instrumental setup of a single coil HSCCC



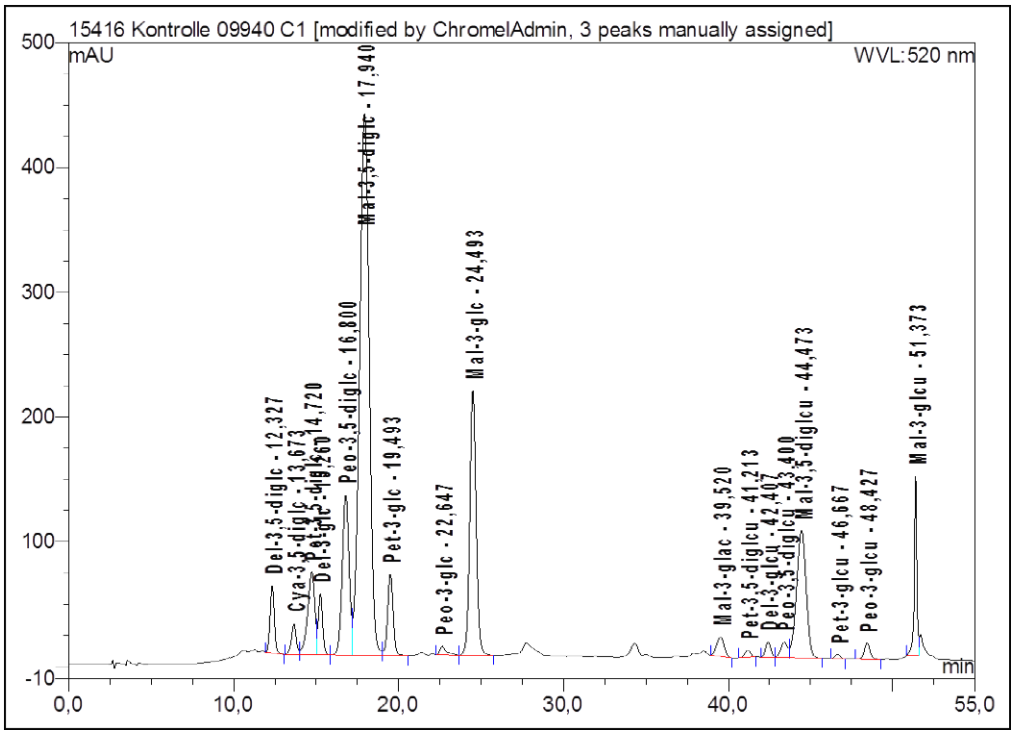
(Source: Picture from Schwarz, *et al.* (2003)).

Figure 26 HPLC machine of FAG



(Source: private picture)

Figure 27 Example of the chromatogram and spectrum



4.2.4 Oxidative capacity

(TEAC - SOP-L-027-1)

Principle

The principle of this method TEAC value (Trolox-Equivalent-Antioxidative-Capacity) is based on comparing the capacity of an artificial antioxidant and water-soluble vitamin E derivative (Trolox®) with the antioxidant capacity of the samples. The addition of

potassium to a solution of ABTS (see below) forms the long-lived radical cation (ABTS +), which at 734 nm has a maximum absorption property. By antioxidants, the radical cation (ABTS +) is destroyed and discoloured the intense blue coloured solution. The discoloration of the sample solution is then a measure of antioxidant capacity, which is expressed in Trolox equivalents. This Method was modified by Dr. Patz after Nikfardjam Pour (2001) based on Rechner (2001) and Miller (1993).

Execution and chemicals

- ABTS (2,2'-Azino-bis-(3-ethylbenzthiazolin-6-sulfon acid) Diammonium salt) (Fluka No. 11557)
- Trolox® (6-Hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid) (Fluka 56510)
- Ethanol
- di-potassium hydrogen phosphate
- Potassium hydrogen phosphate
- Potassium

The device used to do the measurements is a Photometer at λ: 734 nm.
The sample should be diluted according to the total phenol content, for this reason it is recommended to determine the phenols before this TEAC test. With these following data for the PBS-puffer (SOP-L-027-1) it is possible to know the appropriate dilution:

- White wine: 1:10 (Folin >250 mg/L)
- Red wine: 1:20 (Folin < 1.500 mg/L) → 500 µL sample to 10 mL
- Red wine: 1:50 (Folin > 1.500 mg/L) → 200 µL sample to 10 mL

It is important that the samples have a pH between 7.2-7.4, even if a after the dilution. This is ensured by the use of PBS buffer for dilution. It is also import to let the samples prepared to be measured within 10 minutes. Double stipulation is a must.
The extinction differences are formed to calculate the antioxidant capacity, i.e. the absorbance of the blank value is subtracted from the absorbance of the sample and is calculated using a calibration line. The indications of the results are in mmol/l Trolox®, without decimals.

4.2.5 Conductivity

The conductivity in wine can be related to wine stability and its necessity of being cold stabilised, concerned mainly with precipitation of potassium hydrogen tartrate but also calcium hydrogen tartrate (Rankine, 2004).

Principle

For this analysis a device with a conductivity cell of 2 electrodes is used. Its principle is based on comparison measurements; the testing of specific conductibility variations. A

known quantity of potassium bitartrate crystals is added to the wine, and there is an experimental measurement of saturation temperature. Using both methods; mini-contact and saturation temperature together with thermodynamic data, it is possible to know the level of saturation of potassium bitartrate in a wine, and furthermore the tartaric stability of the wine.

Saturation temperature

As already mentioned it is possible to make a statement about the wine potassium bitartrate stability based on the saturation temperature, which could be helpful on making a decision if one should stabilise a wine or not.

The conductivity of a wine is measured in micro-Siemens (µS) at 20 °C with a sensor. Under continuous mixing approximately 4 g per litre of potassium bitartrate is added to the wine, bringing the solution to over-saturation, so that the excess of that salt will precipitate. The conductivity is again measured and the difference before the initial and final conductance is divided by 29.3.

Saturation temperature = µS initial- µS final/29.3

The result will always be positive. Using a table it is possible to make a statement concerning the wine stability. However this mentioned calculation only enables one to have the saturation temperature of KHT. For CaT another calculation is necessary.

The result may be applied to a table that indicates the stability of the sample wine, as follows.

Table 19 Saturation temperature for KHT

	Red wine	**White wine**
Stable	<15°C	<12°C
Unstable	15°C - 19°C	12°C -16°C
Very unstable	> 19°C	>16°C

(Source: Das deutsche Weinmagazin, after Schmitt, 2008)

Differences in white and red are due to the colloidal formation divergence between them. Despite these values safety they are not absolute, wine changes can lead however to tartrate instability and thus precipitation (Schmitt, 2008).

Mini-contact method

The wine conductivity is measured when it is chilled from 0 to - 4°C. Approximately 4 g/L of potassium bitartrate is added under continuously mixing process. A sample super saturation of existent KHT which was dissolved in solution precipitates. In this

case a loss on the conductivity will be noticed by a second measurement that takes place after this crystallisation.

When the dropping of conductivity (at -4°C) drops more than 50 µS/cm the wine can be judged technologically unstable; when the drop is less than 25 µS/cm the wine can be judged stable. In other words; the greater the decrease in conductivity, the more unstable is the wine and the requirement for a treatment (Berta, *et al*., 2003).

Table 20 Stability of wine by mini-contact method

	Red wine	**White wine**
Very stable	30 µS	25 µS
Stable	50 - 60 µS	24 - 40 µS
Unstable	60 - 70 µS	40 - 60 µS
Absolutely unstable	>70 µS	>60 µS

(Source: Manual of Delta Acque Check Stab Alfa, 2008)

Execution

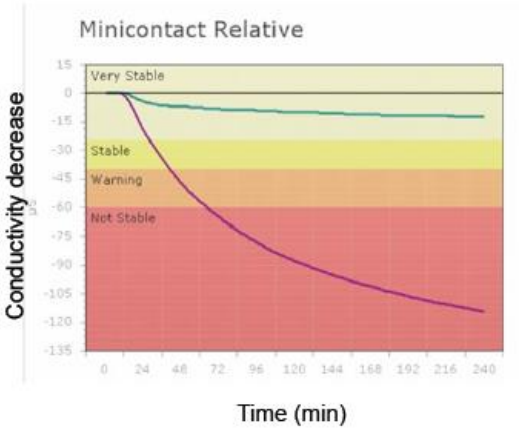
The laboratory instrument used is called "Check stab α2001 Millennium". It is from the company Delta acque – Florence, Italy. The device has automatic calibration of conductivity and automatic compensation for temperature (OICCE and CheckStab).

A beaker glass containing 100 mL of wine sample is inserted in the Check stab device that automatically conducts the following steps: the sample is cooled down to -4 °C and at this temperature the specific conductivity is measured. The following step is the addition of bitartrate crystals of potassium – also automatically, and the instrument waits until it gets the stabilized to do the measurement of the conductivity. The difference between these two measurements gives us an index of the amount of KHT precipitated at -4°C, by the difference in conductivity.

For the saturation temperature method, the first measurement takes place at 20 °C, this temperature is reached mechanically.

"Check stab α2001 Millennium" computer software has a table to simulate by the curves of conductivity the stability of wine, here showed as an example on Figure 28.

Figure 28 Wine stability by minicontact - conductivity



(Source: Vernhet, 2013)

4.2.6 Protein removal and content with "Sardobind S" membranes

The purpose of this analysis is to remove protein from wine. In this case study and project the protein specified is lysozyme, as many times mentioned before to avoid its declaration and allergenicity. Proteins are removed through an ion exchange adsorber membrane that acts like a dye. With this filter it is possible to separate or make the wine free from proteins.

There is no pre-described method for this analysis, so that this description that follows was set as a work instruction in the oenology department of Geisenheim by Dr. Freund with collaboration of the company Sartorius from Göttingen that made available the filter prototype "Sortobind S" for this study.

(Source: private pictures)

Principle

The filter works by adsorption principle. The wine is filtered through a membrane adsorber in well shape, through a dead-end pressure filtration; this filtration separates the protein from wine. This hydrophilic membrane called "Sartobind S" has an anionic substance coated in it, in this case sulfonic acid (S) - a very strong acid with formula (R-CH2-$SO^{3-}Na^{+}$). This membrane is made of reinforced cellulose and this substance applied is the ligand, or Lewis acid. Sulfonic acid belongs to the ion exchanging group, so that there is a strongly acidic cation exchanger. Depending on protein isoelectric point, which determines whereas it is more or less positive charged it will be attracted and stay in the membrane by the negative charge of the sulfonic acid adsorber ligand. Due to a dynamic capacity of 0.6 mg/cm², it is possible to calculate the amount of retained protein by knowing the membrane surface (Freund, 2011).
The membrane has also a microfiltration function with pore size between 3-5µm approximately. The pore size is not relevant for the retention in this study case, as the principle is adsorbance. Furthermore this filter is only adequate to white wine, due to its adsorbance properties.
To settle this experiment some problems needed to be solved

- The flow in the filter must be free of air.
- Wine containing added protein was filtered and after some mL the tubes showed the presence of proteins, meaning the filtration was not properly working, or the filter was "clogged" due to the capacity volume on adsorbing particles related to the filter area. A set of maximal adsorbance capacity is important.
- Find an efficient way to measure the proteins.

Material and execution

Devices

- Filter cartridge with an area of 20 cm²
- Adsorber-membrane Sartobond S (area 20 cm²) - used 5 membranes together to improve the area (resulting in 100 cm²)
- Mini peristaltic pump (at the "frequency" of 70%) or spraying device
- 1000 mL volumetric flasks
- Beaker glasses

Solutions

I. Sodium acetate buffer (pH 5,5) (1M): the activating solution

82g Na-acetate (1M) and 180 mL of 1 mol acetic acid with deionised water

II. Sodium acetate buffer (pH 5,5) (10mM)

The dilution of sodium chloride 1M is with deionised water 1:100

III. Sodium chloride (Eluate)

57, 44 g NaCl (1 M) and 1, 64 g Na acetate (20 mM) – 20 mL 1M Natrium acetate buffer with deonised water in one litre respectively. This is the leaching solution, or eluate.

Bento-solution

This is a ready solution of 12-Molybdophosphoric acid (H3PMo12O40).

Execution of this method

Preparing the filter

The filter has to be prepared by putting all bottom parts together. To remove air from filter Na-acetate (10mM) solution should be pumped and the membranes should be placed by tweezers help. Five membranes will increase the area to 100 cm^2. Filter should be closed and screwed with its upper part of the cartridge. Hoses are than connected to cartridge bottom. The hose end is immersed and placed in a beaker with about 50 mL of Na-acetate buffer. A syringe with around 50 mL of same buffer solution No. II is screwed on the top part of the cartridge and by pressing it one should try to get all the air out of the filter. It is important to note that the lower side of the tube remains immersed in the solution, until the filter has no air on it. So the tubes can be removed and the filter can be closed. This is the way filter can be conserver or be in stand by modus.

Using the filter

The filter should always be flushed in the same way as described above (Preparing the filter) with 50 mL of Na-acetate solution buffer. After flushing the tubes should be empty. In the laboratory of the oenology department there are two possibilities to pump

A) Using a peristaltic pump
B) Using a syringe

Peristaltic pump

Air should be drained and removed from hoses before connecting it to filter by pumping the wine until no air is inside the system anymore. It should work at power of 70%, which equal 66 mL per minute. In our pump there are around 17 mL liquid within the line system. Before the filter 13 mL, after 4 mL and in the filter or the dead volume is 3 mL. Thus, the first 7, or better 10 mL of filtrate are discarded.

Note

The flow is always filled with flushing solution and this is suitable for analytical purposes. When a pure sample is required, 20 mL are enough to reach the whole flow of the line system, this is due to the piping system that is before the filter (13 mL) that means a fourfold change of product in the filter and, which should be sufficient to drain the system completely.

Syringe

After flushing the system should be empty. The syringe should be placed on the upper part of the cartridge. Due to dead volume of 3 mL, a volume of 5 mL of filtrate should be eliminated. Next 10 mL may still be charged, but this is irrelevant for analytical purposes. If the calculated volume of wine is larger than the syringe volume, it can be refilled several times. However, it filter must be always free of air.

Calculation of wine volume

As mentioned before the membrane area in the filter is 100 cm^2 (5 x 20 cm^2). The dynamic capacity of this filter is 0.6 mg per cm^2. This is the area of 60 mg above the filter. If the dynamic capacity is exceed, filtration free of protein cannot be assured.

Example

A wine with 500 mg/L of lysozyme has around 60 mg protein in 120 mL. This means that the filter cannot make the wine free of protein when a volume over 120 mL is filtered.

Controlling the wine filtration

To make sure that the filtration worked properly and protein was retained, samples can be collected in 10 mL test tubes prepared with 1 mL bento solution. If proteins are present even directly at the filling it is visible.
The number of tubes is determined by visual clearance of the sample, which indicates that no protein is coming from the filter any longer.
If a disruption of the calculated amount of proteins happens, then a cleaning process should be carried out.

Controlling the flow rate

Flow rate determination should be done before and after every time one work with this filter. The flow rate is performed with 100 mL of 10 mM Na acetate buffer at a given pump power of 70%. If the value deviates after the filtration more than 40%, then a cleaning process might be initiated (cleaning process is indicated below). The 100 mL of the solution are placed in a beaker glass using a peristaltic pump and passed through the filter. The filtrate is collected in a 100 mL graduated cylinder. Once solution is coming out of the exit hose, a stopwatch is started and when the cylinder has 100 mL stopped. The flow rate is given in mL per minute.

Cleaning the filter - Proteins elution

For the release, or elution of proteins out of the filter it is necessary to use 100 mL solution of 1M NaCl. By filter or syringe means, as already mentioned above, so it can be pumped through the filter. The same procedure with test tubes and bentonite solution (1 mL) should be carried out, and 10 mL of filtrate should be pumped into every tube until it the sample has a visual clearance. If 100 mL solution is not enough to obtain a clear sample, one should carry on with further 100 mL of solution.
After this cleaning process the filter has to be flushed with 50 mL of 10 mM Na-acetate.

Other cleaning process
If the filter has a reduced flow rate or membranes bind capacity, this membrane must be cleaned. Before the cleaning process the membrane adsober module should be cleaned with 1M NaCl solution.
Depending on the application, different cleaning methods and substances are used:
- To depyrogenisation, protein removal and general cleaning of 1 M NaOH for 30 - 60 min at room temperature
- For the removal of hydrophobic contaminants in water 50% isopropanol over 30 min at room temperature
- 1 M H3PO4 for 30 min at room temperature

The following solutions can be supplied to the circuit via a heat exchanger:
- 20 - 50% citric acid in water for 60 minutes at 50 - 60 ° C
- 0.1 M NaOH for 60 min at 30 - 40 ° C
- 1 M H3PO4 over 30 minutes at 40 - 50 ° C
These instructions are after Freund (2011) and Sartobind® Factor-Two Family - Membranadsorber System guide; Installations- und Bedienungsanleitung

Table 21 Membran adsorber System use instructions

1	Remove all air in the filter with Na-acetate buffer	Hose No. 7 with hose +/-
2	Pump 50 mL of Na-acetate buffer – to flush the filter	3 mL in 300 mL: 10 millimol
3	Prepare the tubes with Bento solution	1 mL
4	The pump should be at 70 %	66mL/min
5	Empty all pipes	R in 7
6	100 mL wine (diluting with water if necessary)	Calculate the volume to be filtered
7	Pump 10 mL in every tube prepared with Bento solution	
8	Empty all the small pipes	
9	Pump 100 mL of NaCl solution	
10	Pump 10 mL in every tube prepared with Bento solution	
11	Empty all the small pipes	
12	Na-acetate + acetic acid pumping out 80 mL	To clean
13	Pump the same previews solution in cycle to let the filter in standby/buffer solution	

(Source: These instructions are after Freund (2011) and Sartobind® Factor-Two Family - Membranadsorber System guide; Installations- und Bedienungsanleitung)

4.2.7 Minerals (TRFA)

Principle

Total reflection fluorescence analysis (TRFA) is a method for multi-element determination; with TRFA it is possible to analyse all detectable elements in a sample. For quantification there is an internal standard. An acid chemical extraction takes place in homogeneous and solid samples, while liquid samples can usually be measured directly. In the present study, the TRFA was used to investigate the mineral element content in plant products, with the intention to evaluate its purity (Steinfeld, 2011).

The X-ray fluorescence is based on the irradiation of a sample with primary X-rays, which excites electrons in energy dissipation and return again to the ground state. The emitted secondary X-ray radiation is detected and reproduced in a spectrum. The spectral lines in this spectrum are characteristic of the individual items contained in the sample and allow a qualitative assignment (Bruker, 2008). The sample can thus be analysed for their elemental content. Based on the intensity of the fluorescence radiation, the concentration of each element are calculated (Klockekämper, 1997).

The measurement is done using a spectrometer called S2 PICOFOX and its working principle is that the X-ray beam is generated in the molybdenum tube and the multilayer monochromator reflected, thereby providing a monochromatic X-ray beam. Then it hits at a very small angle of 0.1 ° on a sample carrier, on which the sample is prepared as a thin layer, the beam is reflected here in full. The radiation emitted from the sample fluorescence is detected by an energy dispersive detector. There is a special feature that lies between the detector and sample holder in a distance of only 1.5 mm, so the detector can detect the X-ray fluorescence radiation with very high efficiency and sensitivity of the method is increased (Brucker, 2008 and Steinfeld, 2011).

Execution and chemicals/device

The spectrometer consists of three modules, X-ray tube, monochromator and detector. An important position in this building is occupied by the monochromator. Here, the radiation of X-ray tube is filtered and modified in the spectral distribution and geometry. The X-ray tube and monochromator together form the excitation module. Using a diaphragm system in the monochromator is set to the angle of incidence and on the multi-layer so that only the radiation of interest is reflected. It also uses a filter made of metal foil in order to suppress low-energy X-ray photons, or else this would happen in the total reflection multilayer (Brucker, 2008). After detectors perception of beam reflection from the sample fluorescence, a spectrum is

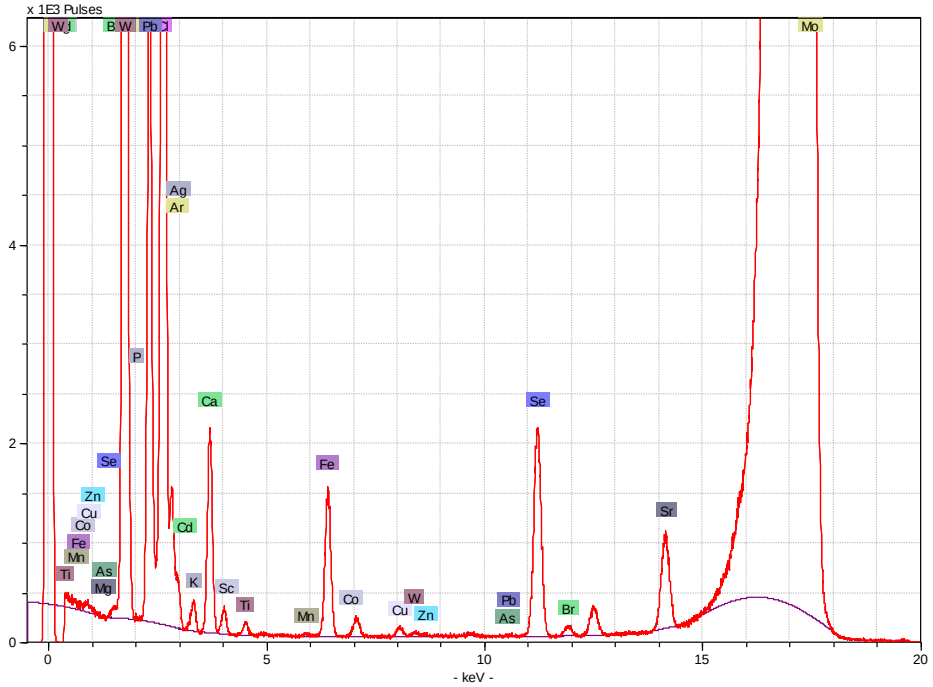
created and the spectra analysis is performed automatically by the software (Brucker, 2008).

Preparation of samples

- 10 mg of sample was weighed into an Eppendorf reaction vessel with 2 mL of double distilled water
- 100 L of a 0.01 g/L selenium solution (internal standard) was added and homogenized.

Resulting suspension is applied 2 times; 5 mL to a quartz sample holder; with double determination.

Figure 29 Example of spectra of TRFA



4.2.8 Enzyme-Linked-Immunosorbent Assay (ELISA)

This is the method chosen to detect the presence or absence of fining material residues in wine. In this study there are different ELISA-tests used throughout this work, which have been undertaken in different laboratories as well; in the city of Darmstadt - with specific kits developed from a company called r-biopharm and in Hamburg – developed exceptionally for this project with specific wine matrixes. Each one of them will be explained with more details bellow.

Immuno-Assay with ELISA kits (r-biopharm)

This ELISA is an in vitro quantitative sandwich Enzyme Linked Immunosorbent Essay test. The kits are called Ridascreen® Fast and with them it is possible to measure casein, lysozyme and egg white proteins; approximately 54% ovalbumin, 12% ovotransferin and 11% ovomucid (R-Biopharm, 2008 and 2010).

Principle

The basis of the test is the antigen-antibody reaction for the quantitative analysis of casein in wine. All reagents required for the ELISA, including standards, are contained in the test kit. The wells of the microtiter strips are coated with specific antibodies against casein and egg white proteins. This coating process is done in the laboratories from the company, for this reason they are "ready kits" for the every specific protein to be detected. By adding the standard or sample solution in the wells, present specific proteins will bind to the specific antibodies. The result is an antibody-antigen-complex. In washing step components not bound are removed. The antibody conjugated to peroxidase is added. This antibody conjugated is bound to the Antibody-Antigen-complex. An antibody-antigen-antibody (sandwich) complex is formed. Any unbound conjugate is then removed in a second washing step. The detection of casein/egg white proteins takes place by adding substrate/chromogen solution. The enzyme conjugated converts the chromogen into a blue product. The addition of a stop solution, in this case an acid, leads to a colour change from blue to yellow. The measurement is made photometrical at 450 nm. The absorbance is proportional to the casein/egg white proteins/lysozyme sample (Lacorn *et al*., 2009).

The sensitivity of these tests is given by the Limit of Detection (LOD) or the lowest detectable level for the Ridascreen® Fast casein/egg white proteins/lysozyme that can be distinguished from zero matrices. They are different for all the three test-kits, such as the Limit of Quantification (LOQ) or the lowest/highest concentrations that can be determined in a sample with acceptable precision (repeatability).

Table 22 ELISA test-kit limits of detection and quantification

	LOD (ppm)	LOQ (ppm)
Casein	0.12	0.5
Egg white proteins	0.27	0.5
Lysozyme	0.02	0.05

(Source: R-biopharm Ridascreen® Fast, 2011)

Table 23 ELISA kit Standard concentration –Egg proteins

Standards for egg proteins	ppm
1	0
2	0.5
3	1.5
4	4.5
5	13.5

Table 24 ELISA kit Standard concentration -casein

Standards for Casein	**ppm**
1	0
2	0.5
3	1.5
4	4.5
5	13.5

Specificity of the test for

- CASEIN: The monoclonal antibodies specially detect α-, β- and κ-caseins of cow's milk. No cross reactivity to β-lacto globulin, such as no cross reactivity to caseins of other animal species (sheep, goat).
- EGG PROTEIN: The specific polyclonal antibodies detect antigens from egg white proteins – mainly ovalbumin, ovotransferin and ovomucid.
- LYSOZYME: The antibodies specially detect lysozyme of hen's egg.

Execution and chemicals

- 1 x microtiter plate with 48 wells (6 strips with 8 removable wells each). They are already coated with anti-casein/lysozyme/egg white antibodies. The microtiter-plates are irradiated to provide an increased binding affinity for hydrophilic proteins. The binding is non-covalent.
- 5x Standards (1.3 mL). They start from 0 ppm, (S1; zero standard). For casein and egg the standard concentrations are the same (0/0.5/1.5/4.5/13.5 ppm). The standard concentration for lysozyme is different (0/0.050/0.100/0.200/0.400 ppm). All in aqueous solution ready to be use. The standard solution is a buffer containing the analyte and different additives to stabilize the analyte-protein. Every antigen needs different conditions and so the additives differ from assay to assay.
- 1 x Conjugate (0.7 mL). Peroxidase conjugated antibody, concentrate
- 1 x Substrate/Chromogen (10 mL). Stained red. The substrate is hydrogen peroxide and the chromogen is TMB (tetramethylbenzidine). The reduced form of TMB is colourless; the oxidized form is blue (neutral pH-value) or yellow (acid pH-value).

- 1 x Stop solution (14 mL). Containing 1 N sulphuric acid
- 1 x Extraction buffer (125 mL). A 20fold concentrate. The extraction buffer is a phosphate-buffer with different additives, optimized to extract at 60°C.
- 1 x washing buffer (100 mL). A 10fold concentrate. The washing buffer is a phosphate-buffer containing synperonic. This increases the moistening of the wells and therewith the washing effect.

Figure 30 ELISA Test-Kit Ridascreen®

(Source: Photo from R-biopharm)

Further material/devices required (not contained in the kit)

- Microtiter plate spectrophotometer (450nm)
- Water bath
- Ultra-Turrax or mixer/homogenizator
- Graduated pipettes (more canal pipette and multivolume pipette). Variable 20 µL -200 µL and 200 µL-1000 µL micropipettes.

The execution of this method is as follows:

The first part is the sample preparation and extraction. As wine, normally and, in our case does not have to be filtered or grinded; it is used directly.

- 1 mL of wine sample to 19 mL diluted extraction buffer (10 minutes at 60°C extraction).

The second part is the test procedure (Figure 18):

- Add 100 µL of standard or sample and incubate for 10 minutes at room temperature
- Pour the liquid out of the wells. Wash 3 times with washing buffer
- Add 100 µL of the diluted enzyme conjugated to each well, mix gently and incubate 10 min at room temperature.
- Pour the liquid out of the wells. Wash 3 times with washing buffer
- Add 100 µL of substrate/chromogen, mix gently and incubate for 10 minutes at room temperature in the dark
- Add 100 µL of stop-reagent- mix gently. After 10 min measured photometrically at 450 nm against an air blank

Figure 31 ELISA procedures in pictures with Test-kits

standard and sample washing buffer enzyme conjugated stop-reagent

(Source: Photo from R-biopharm)

The assay result is specific, sensitive, and takes around 50 minutes. This can be done in a small laboratory, which means it is flexible. There is, though, the need of a special spectrophotometer as to be seen in Figure 32.

Figure 32 Photometrical measurement of ELISA

(Source: Photo from R-biopharm)

4.2.9 ELISA developed and carried out in Hamburg University

For these tests a special matrix for wine was developed, namely very sensitive analytical methods are necessary to detect possible fining agent residues in wine.

The immunization of host animals with the fining agents used in the cellar led to polyclonal antibodies, which were used to develop diverse ELISA methods: Indirect ELISA for casein (0.1 ppm LOD) and ovalbumin (LOD 0.006 ppm) can be used for white and low phenol red wines; Indirect ELISA for lysozyme (LOD 0.006 ppm) for white and red wine; Direct sandwich ELISA for ovalbumin in white and red wines (LOD 0.005 ppm); Indirect sandwich ELISA for casein for white wine (LOD 0.01 ppm) and red wine (LOD 0.1-0.3 ppm). To achieve the proper LOD the scientists used to analyse the data a 4-parameter-regression with a softwear called SoftMax Pro 5.4 (molecular devices), together with precision-profile to follow the variability of error along the curve. The measuring range is determined by setting the threshold value of 20% for the relative error (AIF 16330 N, 2012 and Deckwart, 2012).

- an **indirect-ELISA** was developed to detect **ovalbumin and casein in white wines** and for low-phenol-containing red wines
- a **direct-sandwich-ELISA** to detect **ovalbumin in red and white wines**
- an **indirect-ELISA** for **lysozyme for white and red wines**

- an **indirect-sandwich-ELISA** for **casein in red and white wines**

These different methods were aiming to achieve a higher specificity of polyclonal antibodies raised against every fining agent that were used for assay development in this study. From the decision of antibody to be used to method of ELISA to be applied, everything has been done in the laboratories of Hamburg- project partner of this study (AIF 16330 N, 2012 and Deckwart, 2012).
All methods can be found in the bulletin of:
AiF 16330 N *„Reduktion des Gehaltes allergener Weinbehandlungsmittel im Endprodukt Wein durch technologische Verarbeitung“ – Schlussbericht 2012* – (AIF 16330 N, 2012).

4.2.10 Sensory analysis

To study the influence of each fining material and the filtering methods on the final product, wine, sensory evaluations have been done in the sensory analysis laboratory of the research centre in Geisenheim. These analyses are aimed to demonstrate the effect of all practices applied. This part of the work is displayed in year 1 and year 2 to facilitate the division and understanding of each tasting.

4.2.10.1 First year wines

Mueller-Thurgau and Regent (Wines No. 1 and 2 of result chapter)

Pre tasting principle

All variants of wines were tasted together in the form of pre-tasting to define and classify them for future tastings, this have been done within the group of researches and partly also with a larger group including other experienced tasters.
In this pre-evaluation for the first year wines (Regent and Mueller-Thurgau, wines No. 1 and 2) panellists with experience in the area, had the unanimous decision that it was not possible to perceive the difference between the fining agents within the wines fined with the maximal concentration. Notwithstanding for statistic confirmation on this decision a tasting was held, described below – Simple Ranking Test: Friedman’s analysis.
As many times previously mentioned, the wines were fined with high doses of fining material; doses that are not normally used in cellar practice. The reason for these high doses is to test if one can ensure that even with values above normal; within the idea of worst-case scenario, the wine can be safe for consumers. In fact if not by accident, these doses would normally never be used in practice. Therefore it was difficult to classify it in groups for tasting and for the same reason more research on following

years were done with lower doses to bring wine closer to winemaking reality. This test has been done together with Miss Germain, a master student of Geisenheim.

First tasting - Simple Ranking Test: Friedman's Analysis
Objective: To determine whether significant differences exist among the fining agents for the intensity of given attributes.

Test design:
Five white wine and five red wine samples were evaluated by a panel of **15 to 20** students and/or professors from Geisenheim research centre. Each subject received the five samples of white wines, coded with three digit codes and served in a random order. The same procedure will be followed for the red wines.

Samples:

1. Control
2. Ovalbumin
3. Casein
4. Potato Protein
5. Pea Protein

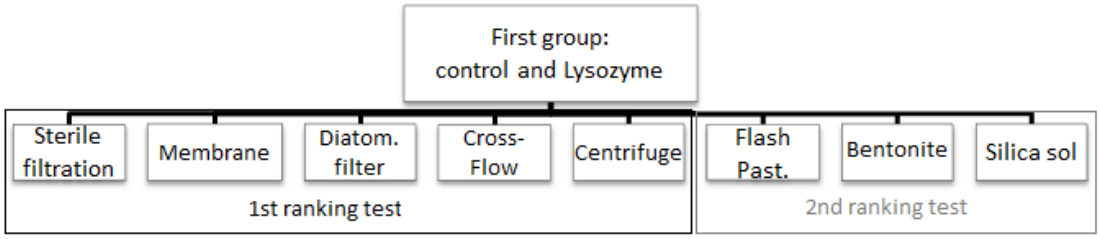
Panellists have been asked to rank first the white wines and then a red wine in order of intensities for the following attributes:
Rank 1 to the lowest intensity and rank 5 to the highest intensity of:

White Wine	Red Wine
1. Aroma: Fruity intensity (Peach, apricot, pineapple, lemon, melon)	1. Aroma: Fruity Intensity (Plum, cherry, blackcurrant)
2. Flavour: Fruity/Floral intensity	2. Bitterness
3. Palate: Body (Light to Full)	3. Astringency
4. Bitterness	4. Preference
5. Preference: Rank 1 to 5	

Second tasting - Simple Ranking Test: Friedman's Analysis
Objective:
To determine whether significant differences exist between the filtration methods within every used fining agent; based after personal preference of the taster in aroma and flavour intensity.
Test design:

Due to the great number of samples the tasting was divided firstly into wine type; white and red. In this way this tasting had a result of 8 appointments; 4 for each colour. Secondly it was divided in 4 groups according to the fining agent origin, one appointment per origin of the fining agent. Each group has two ranking tests, which has been done on the same appointment. The first ranking has only filtering methods, while the second has the other methods used in this study as to be seen on following diagram example.

Diagram 4 Diagram of first tasting appointment



Wine samples were evaluated by a panel of 15 to 20 students and/or professors from Geisenheim research centre. Each subject received maximal five samples wines per ranking test, coded with three digit codes and served in a random order. The same procedure will be followed for the red and white wines.

First group - non fined wine, or namely control and lysozyme have been tasted. Lysozyme was grouped with control because of its classification; this is not a fining material. Control wine and Lysozyme should here represent the samples that suffer no or less effect from fining, meaning that if any differences in these wines are found they should be almost purely due to the difference on the filtering method.

The second group - it is formed by different animal proteins; ovalbumin, isinglass and gelatine.

The third group - it has only milk proteins; whey protein, casein and SensoVin (potassium caseinate).

The fourth group - it has vegetal proteins from potato and pea and also a synthetic agent named PVPP.

It is important to remember that even though the proteins have been classified into groups this tasting is purely aimed to determine with a ranking test the differences between technologies used after each fining procedure.

4.2.10.2 Second year wines

Triangle sensory test

(Wines No. 3 to 9 of results chapter)

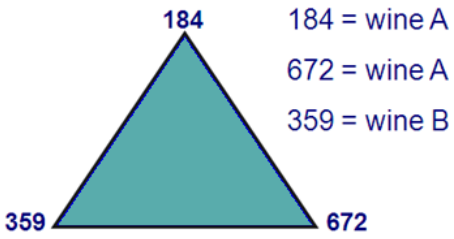
This test is based on Bisson, 2013 description of triangle test. For this test the tasters were offered three wines. Two of the wines are identical and one is diverse. The taster is communicated about this and is asked to recognize the wine that is diverse. Finally the taster is asked to choose the preferred wine, which is not described by Bisson, but is a common practice for triangle test at the research centre of Geisenheim.

Objective:

To determine whether significant statistic can be applied for recognized wines as well as if there is any further significance on the preferred wine.

A statistical table is used to determine if the percentage of correct answers is significant or not, that is, is improbable to have occurred by chance. A random three digit codes numbering scheme was used so that the tasters are not influenced by some numerical consistency of the analysis.

Figure 33 Triangle sensory wine test – an example



(Source: Bisson, 2013)

4.2.11 Clinical trials from Technical University of Munich

This medical research was conducted in the Clinic and Policlinic for dermatology and allergology in Biederstein Technical University Munich (Brockow, 2011).

Patients with milk and egg allergy were recruited for these trials and participation in the study is voluntary.

Important to be mentioned here is the extremely challenging task of recruiting patients for the test, maybe this being one of the leading obstacles on allergy studies. The Department has a database of the precedent five years before the beginning of this study. This database had patients in which food allergy was detected and tested with positive serum IgE assay. From 16,461 patients 1,931 were children and adolescents (0-18 years) and 14,530 adults, 331 were positive IgE for egg or milk and from them only 161 are allergic to cow's milk. Of these 331 patients only 32 adult patients suffered a clinically manifest food allergy to egg and/or milk (only one person presented both an allergy to hen's egg and cow's milk). Finally only 10 patients were included in the test, due to lack of time, pregnancy, strict alcohol abstinence, among others. Each one from these 10 reported a positive anamnesis after ingestion of

chicken egg or caw's milk, as well as for skin prick test. The patients were eight women and two men ranging from 26-63 years of age (mean age 42).

This is the clinical routine skin test for detection of sensitization to food allergens.

Type and duration of use: with a needle stung through a drop of allergen solution superficially into the skin; positive reactions are seen after about 15 minutes with a redness, itching and wheal at the test site, which disappear after 1-3 hours. The following day, can occur locally redness, itching and mild swelling. To be considered a positive result in this study a wheal has to have a diameter greater than 3 mm with accompanying erythema greater than 5 mm compared to the negative control. The positive control was carried out with 0.1% histamine and the negative control containing 0.9% NaCl.

Investigational: the test allergens are incorporated into an aqueous solution or as unmodified foods. The starting materials used are fined and not fined wines, plus its fining agents (diluted and undiluted), milk, egg, and a standard to check the allergy tendency.

Benefits and risks of testing: this test can be determined whether these allergens could have an influence on the initiation or severity of symptoms. The test allergens can trigger skin reactions such as itching, redness, or hives. Moreover, there may be conjunctivitis, runny nose or hives asthmatic symptoms or other systemic reactions are extremely rare. Such events are very rare and usually only in the first hour after the test.

Taking of a blood sample

Benefits and risks of testing: a blood sample is taken to detect sensitisation to food allergens and to test the allergenicity of fined wine (30mL whole blood in adults). Blood withdrawal can lead to local pain (restricted to the site of blood sampling) and a hematoma formation (haematoma, "bruise").

The oral provocation

The oral provocation is performed with fine and not fined wine. To avoid confusion, this provocation is carried out in a "double blind" form. This means that during the assessment of the responses judged neither patient nor physician know what is tested at the moment - the treated wine or a neutral wine without fining agent (placebo). Only after the completion of the provocation, when all reactions are interpreted, it

comes to the "unblinding". This form of double-blind study has been proven in clinical practice and deemed "gold standard". In the present study, the DBPCFC was performed according to the recent guidelines of the European Academy of Allergology and Clinical Immunology (EAACI).

Type and duration of use: the oral provocation takes place in two "test blocks" for each one day. On the first day patients get the wine to be tested in an ascending dose (amount), starting with a very small amount of 0.1 mL, if no symptoms are felt, after 30 minutes, a larger amount of wine is given (10 mL). If after another 30 minutes nothing occurs, again, no complaints, other 189 mL of wine are given for women and 289 for men. Thus, the total dose was 200 ml in women and 300 ml in men. In case of possible responses patient will be in observation for about 2 more hours. Before the provocation test volunteers has to draw blood for laboratory tests. During the test period, patients must remain on the station. Only after an explicit permission from the doctor they may be leave the station. On the first day of the testing takes about 4 hours. On the second day patients come again for a repetition on oral provocation. This is the same provocations test that is repeated, but only with another wine. The oral provocation with fined (*verum*) and no fined (placebo) wine was conducted on two days, the time interval between treatment and placebo testing was at least 48 hours.
Investigational: the wines are to be tested without changes.

Benefits and risks of testing: this test can determine whether patients can tolerate wine that has fining agents on the basis of cow's milk and egg protein.
Despite the onset of low drug doses, it can in particular cases lead to general reactions with various symptoms (e.g. urticaria/hives, generalized rash, cardiovascular reactions, nausea/vomiting) or even come to a shock reaction. Therefore, while the tests are being undertaken patient may not leave the station without the express permission of the physician. Since during the test it may be possible (as in antecedent cases of severe reactions), a venous cannula, it may rarely occur at the puncture site a haematoma (bruise), local pain or inflammation.
All methods can be found in more details on the bulletin of:
AiF 16330 N *„Reduktion des Gehaltes allergener Weinbehandlungsmittel im Endprodukt Wein durch technologische Verarbeitung“ – Schlussbericht 2012* – (AIF 16330 N, 2012)

5 Results and Discussion

In this chapter all results are directly followed by discussion. Wines used for this study are displayed at the end of this chapter.

Results order

This chapter displays firstly all ELISA (5.1) results; the ones done in Hamburg as well as tests done using test kits. Results are divided by trial year and further by each protein. The ELISA is followed by the clinical tests (5.2) undertaken in Munich. Wines were previously tested in vitro before in vivo to assure the quantity of residues to be safe to patients.

A sequence of results on wine phenols (5.3) is then presented, since they are the main reaction partner of fining agents. Turbidity helps to understand the effectiveness of fining (5.5).

Analysis on wine and fining agent minerals are done to check its purity or the presence of further contamination or residues (5.6).

Further analysis are helping to present the efficiency of fining agents or helping to find and understand alternatives; colour of wine (5.7) and sensory analysis (5.8).

Wine conductivity results are related to interaction of lysozyme and metatartaric acid, alongside with wine colloidal solution better comprehension, in relation to some fining agents. Finally an attempt of removing or/and quantifying lysozyme is presented by using an adsorbance laboratory-scale-filter (5.10)

5.1 ELISA-test – detection of fining residues

The following results are divided by first, second and third trial, time wise a trial per year.

5.1.1 First trial wines (2010)

Results from Hamburg's ELISA (in vitro)

Ovalbumin

To interpret following results one should take into consideration that Table 25 is divided in white and red wines and further divided in three columns which show the three steps of sampling used in this work; A1 directly after fining, A2 separation methods and A3 sterile filtration "EK".

Table 25 ELISA-Hamburg - residues measurement for ovalbumin MT and Regent

Fining Albumin Simple [ ]: 16 g/hl (160 ppm) Double [ ]: 32 g/hl (320 ppm)	**Residues Measurements [ppm]**					
	White wine: Mueller-Thurgau (by indirect ELISA)			Red wine: Regent (by direct sandwich ELISA)		
Dosage	Method			Method		
	After fining A1	**After Membrane** A2**	**After EK* A3**	**After fining A1**	**After Membrane** A2**	**After EK* A3**
Simple []	2.9	<0.25 (0.06)	<0.25 (0.06)	<0.25 (0.03)	<LOD	<LOD
Double []	11.5	<LOD	<0.25 (0.06)	<0.25 (0.04)	<LOD	<LOD
		After K-100			**After K-100**	
Simple []	0.46	<LOD	<LOD	<0.25 (0.02)	<LOD	<LOD
Double []	8.3	<0.25 (0.01)	<LOD	<0.25 (0.01)	<LOD	<LOD
		After Cross-flow			**After Cross-flow**	
Simple []	3.8	<0.25 (0.1)	<0.25 (0.01)	<0.25 (0.03)	<LOD	<LOD
Double []	-	-	-	<0.25 (0.01)	<LOD	<LOD
		After fine Diatomaceous earth			**After fine Diatomaceous earth**	
Simple []	2	<0.25 (0.09)	<LOD	<0.25 (0.04)	<LOD	<LOD
Double []	7.7	**0.36**	<0.25 (0.01)	<0.25 (0.06)	<LOD	<LOD
		After Centrifuge			**After Centrifuge**	
Simple []	2.6	**2.11**	<LOD	<0.25 (0.07)	<0.25 (0.1)	<LOD
Double []	7.6	**3.74**	<0.25 (0.04)	<0.25 (0.08)	<0.25 (0.2)	<LOD
		After FP			**After FP**	
Simple []	4.7	**16.83**	<0.25 (0.01)	<0.25 (0.1)	<LOD	<LOD
Double []	26	**41.13**	**0.55**	<0.25 (0.05)	<LOD	<LOD
		After Silica sol			**After Silica sol**	
Simple []	2.5	**1.60**	<0.25 (0.01)	<0.25 (0.09)	<0.25 (0.04)	<LOD
Double []	20.2	**3.75**	<0.25 (0.04)	<0.25 (0.1)	<0.25 (0.03)	<LOD
		After Bentonite			**After Bentonite**	
Simple []	1.4	<0.25 (0.03)	<LOD	<0.25 (0.02)	<LOD	<LOD
Double []	4.4	<0.25 (0.1)	<LOD	<0.25 (0.01)	<LOD	<LOD

EK*: Sterile filtration- ⌀ 0.45 µm. **Membrane cartridge ⌀ 0.45 µm LOD Limit of Detection. LLOQ Lower Limit of Quantification

(Source: modified from AIF 16330 N, 2012)

Table 25 of ovalbumin fining agent has on the first column, for **white wine Mueller-Thurgau**, 7 samples with positive ELISA;

1. Filtration with fine diatomaceous earth method on double concentration has 0.31 ppm remaining after filtration. There is a reduction of 99.9% of ovalbumin even in the worst case or double dosage.
2. Centrifugation method on simple and double concentration has 2.1 and 3.7 ppm, respectively, remaining after centrifugation. There is a reduction of 98.7% and 98.9%, respectively.
3. Flash-Pasteurisation on simple and double concentration has 16.83 ppm and 41.13 ppm of ovalbumin, respectively, that remains in wine. These values make a reduction of 89.5% and 87.1% of ovalbumin after flash pasteurisation.
4. After silica sol co-fining on simple and double concentration there is 1.60 ppm and 3.75 ppm present on analysed wines, respectively. The reduction was of 99% in both cases after the use of 50ml/hl of silica sol.

Nevertheless all 7 above mentioned samples, apart from one, shows results under the LOD for when wine passes through a sterile filtration subsequently. The only positive sample after sterile filtration is double concentration after pasteurisation. From the added value of 320 ppm there is a residue of 0.55 ppm.

Important to be mentioned on this study is that in none of the wines, especially the whites, the same level of fining agent is equal directly after the fining process, “A1” see Table 25 and Table 26. Nonetheless there is a mean of 7 ppm. This is due to sampling; samples were taken with a plastic hose by suction. If particles were in or were brought to suspension during sampling they are consequently in sample, although hose were marked evenly to take the sample from the same level and samples were taken in triplicate. The method chosen for sampling the wines mimic a standard racking at a commercial winery (Rankine, 2004; Ribéreau-Gayon *et al*. 2006 and Troost, 1985).

The mean of 7 ppm, for both simple and double concentration for white wines, directly after filtration is already a very low value if compared with added quantity. It shows that fining reactions of positively charged ovalbumin that attaches and absorbs negatively charged substances such as phenols, and above gravity alone makes a great reduction of the fining content to form a flocculent precipitate in wine, as seen in other studies (Lacorn *et al*., 2011 and Webber *et al*., 2007). Not to forget that the decanting time was 24 hours, which was the chosen set up time for this “worst case scenario” study. In wineries this decanting time is normally longer, meaning probably better settling. Other studies with fining agent normally let it settle for one week, and some literature mention the same interval for racking wines from fining deposit. Furthermore in practice most wine cellars have a racking-valve used to properly and

carefully separate decanted wines from settled material (Cosme *et al*., 2009; Rankine, 2004; Troost, 1988; Ribéreau-Gayon, 2006 and Margalit, 2004).

Questions have been posed concerning the elevated residue on flash-pasteurised samples. Proteins, and other wine substances might change, aggregate or denaturise with high temperatures, making the reading likewise higher.
Ovalbumins are heat-labile proteins, furthermore previous studies showed structural changes caused by heating ovalbumin under different time and temperature conditions and the influence of heat on the immunological reactivity of ovalbumin. A study found out a great increase of exposed sulfhydryl groups and surface hydrophobicity after denaturing treatments. Additionally the authors of this study say that by using ELISA denaturing or partially denaturing treatments have an influence on immunochemical reactivity, which rises and could lead to an over- or underestimation of the actual protein level (Rumbo *et al*., 1996).
On this study flash-pasteurisation might change the protein conformation or cause a partial denaturation due to high temperatures of 72°C during ≈20s causing in addition elevated ELISA readings. Further literature says that heat treatments may lead to the appearance of protective polysaccharides that avoids full agglutination of the proteins present (Ribéreau-Gayon *et al.*, 2006).
Ribéreau-Gayon *et al.* (2006) says that a consequence of heating is the formation of protective colloids, he further mentions that there is not sufficient studies on this area. He also says that red and white wines that have been heated and re-cooled generally have properties similar to those produced by adding a protective colloid. Most important and of great relation to this study is his mention that in some wines, particle sedimentation is slower, filtration is more problematic and the flocculation of gelatine and ovalbumin for fining purposes becomes nearly impossible (isinglass and casein are less subtle to heating).

Nevertheless a repetition of flash pasteurisation was done with another wine. On this repetition results are negative for samples directly after fining and after flash-pasteurisation, but positive after EK-filtration. It can be seen on Table 35 with further discussion.

The positive value for centrifuged white wines may be explained though particle size and centrifuge mechanisms. Physical forces weren't strong enough to separate the proteins that are in colloidal solution. The electric charge and solubility are also factors in the stability of colloidal particles in wine. The status of the electric charge is dependent mainly on pH. Flocculation is induced only when electric charges are neutralised, or at its isoelectric point - pI (Farbas, 1988).

As for flash pasteurisation, this trial has been repeated and can be seen on table Table 35.

Silica sol suspension was cited before as being an useful adjuvant to gelatine fining. The silica sols are predominantly used to accelerate fining processes as well as to eliminate excess fining agent, improving filterability of the wine (Rankine, 2004; Ribéreau-Gayon *et al*. 2006 and Troost, 1985). In this case of Table 25 with ovalbumin it has low influence, not interacting enough to be able to remove this protein.

The second column at Table 25, of ovalbumin fining agent, has no sample with residues for red wine Regent, all results are under the LOD.
Already directly after fining and after any filtration all the values were under 0.25 ppm. This can be explained by the phenol content in red wine that is over 10 times higher than in white, therefore higher affinity reactions occurs. Likewise others study had the same results (Lacorn *et al.*, 2011 and Flanzy, 2000).
Control wines are not included in the table, but have been equally tested. No residues were found at any of the control wines, meaning that no cross-contamination occurred.
Results are shown in values that equal or are higher than 250µg/L. Detailed original table is to be found in annexes chapter, where statistic is displayed.
The decision of using 0.25 ppm as parameter of presentation here is due to labelling law. Since July 1st, 2012 the European Commission accepted the proposed limit by OIV resolution through implementing Regulation 579/2012. Therefore all wines containing fining residues from ovalbumin, lysozyme or casein should be declared on the label if the quantity found in it is over or equals 0.25 mg/L (Christmann *et al.,* 2012).

Casein
Table 26 of casein fining agent has on the first column, for **white wine Mueller-Thurgau**, 2 samples with casein residues:

1. Flash-Pasteurisation on simple and double concentration with 2.83 ppm and 9.48 ppm of casein respectively that remains in wine.

Table 26 ELISA-Hamburg - residues measurement for casein. MT and Regent

Casein Simple []: 40 g/hl (400 ppm) Double []: 80 g/hl (800 ppm)	**Residues Measurements [ppm]**					
	White wine: Mueller-Thurgau (by indirect ELISA)			Red wine: Regent (by indirect sandwich ELISA)		
Dosage	**After fining K1**	**Method K2**	**After EK* K3**	**After fining K1**	**Method K2**	**After EK* K3**
		After Membrane**			**After Membrane****	
Simple []	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Double []	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		After K-100			**After K-100**	
Simple []	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Double []	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		After Cross-flow			**After Cross-flow**	
Simple []	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Double []	-	-	-	<LOD	<LOD	<LOD
		After fine Diatomaceous earth			**After fine Diatomaceous earth**	
Simple []	LOD<x<LLOQ	<LOD	<LOD	<LOD	<LOD	<LOD
Double []	0.9	<LOD	<LOD	<LOD	<LOD	<LOD
		After Centrifuge			**After Centrifuge**	
Simple []	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Double []	0.3	<LOD	<LOD	<LOD	<LOD	<LOD
		After Flash-Pasteurisation			**After FP**	
Simple []	<LOD	**2.83**	<LOD	<LOD	<LOD	<LOD
Double []	LOD<x<LLOQ	**9.48**	<LOD	<LOD	<LOD	<LOD
		After Silica sol			**After Silica sol**	
Simple []	LOD<x<LLOQ	<LOD	<LOD	<LOD	<LOD	<LOD
Double []	1.4	LOD<x<LLOQ	<LOD	<LOD	<LOD	<LOD
		After Bentonite			**After Bentonite**	
Simple []	0.4	<LOD	<LOD	<LOD	<LOD	<LOD
Double []	LOD<x<LLOQ	<LOD	<LOD	<LOD	<LOD	<LOD

EK*: Sterile filtration- ⌀ 0.45 µm; **Membrane cartridge ⌀ 0.45 µm; LOD Limit of Detection. LLOQ Lower Limit of Quantification

(Source: modified from AIF 16330 N, 2012)

Almost all wines show negative results already before any filtration –K1, meaning that the fining and racking is in some cases already sufficient for a negative result, or for no

residues in wine on this study case. Casein flocculates very quickly easy to be seen visually. Caseins are insoluble at low pH, thus the pH of wine explains why it precipitates. The proteins have also hydrophobic or non-polar regions that are exposed when caseins denature at wine pH. These regions can interact with phenolic compounds and other components (Horne, 2002 and Fox *et al.,* 1998).

All white wines fined with casein show a negative result when those are as a final point filtered with "EK"-sterile filter. Even directly after fining almost all wines have extremely low values or are negative.

The positive results for flash-pasteurised samples here have the same explanation as for ovalbumin, mentioned before. Furthermore and therefore there is a repetition of this trial on table Table 34.

The right main column of Table 26, red wine Regent, has not detectable allergens results for ELISA, in none of the samples casein has been found.
With both results, whites and reds, one may conclude that in this study, apart from flash pasteurisation sample with no filtration, all other filters are efficient enough for reducing fining agent proteins from wines to extremely low levels or even to not detectable ones.

Lysozyme
For the first-trial-wines treated with lysozyme and passed through many different methods of filtration and separation, the indirect-ELISA shows results that are all positive and far over 0.25 ppm for white and red wines. Only the sample: "simple concentration "or 500 ppm + bentonite fining followed by EK filtration (in both red and white wines) the concentration was under 0.25 ppm. Filtrations are not capable to decrease lysozyme, only bentonite followed by filtration.

These results were not a surprise since lysozyme is an additive and not a fining agent. This enzyme has low MW and is well known to be reduced or removed by bentonite due to its opposite charge and has been showed in previous studies to remain to a certain quantity in wine (Webber-Witt, 2009 and Lacorn, 2009).

As previously showed in literature chapter, other studies showed that physical treatments, such as centrifugation, filtration at 0.45 µm and cooling at -5°C for 120 hours, did not cause any variation in enzymatic activity, meaning that the protein was still present in the solution. Not to forget that the maximum stability and activity for lysozyme is establish at pH values lower than 7.0, specifically, in the range of 2.8-4.2,

which is coincidentally the pH range of most wines (Pitotti *et al.,* 1991). Subsequently if a relation is made between previous studies and this study results, the best option is to declare it on labels or to use other alternatives to avoid the malolactic fermentation.

SensoVin (mixture agent containing potassium caseinate)

Table 27 shows negative results apart from one sample for simple concentration. The positive sample is flash-pasteurised wine with 1.4 ppm, but after sterile filtration the result is no longer detectable. Wines that have double concentration have two samples that have detectable allergens; the flash-pasteurised wine with 1.4 ppm, plus the silicate gel with 0.6 ppm. Nevertheless both of them are negative when the wine passes through a final sterile filtration. Wines treated with bentonite have a detectable amount lower than the OIV limit of 0.25 ppm.

Table 27 ELISA-Hamburg - residues measurements of first trial for SensoVin

White wine - Mueller-Thurgau						
SensoVin - simple dose in ppm				**After sterile EK**		
	Mean in ppm	SD	CV in %	Mean in ppm	SD in ppm	CV in %
Sterile filtration (K-100+EK)	<LOD	-	-	<LOD	-	-
Cartridge membrane 0.45 µm	<LOD	-	-	<LOD	-	-
Centrifuge	<LOD	-	-	<LOD	-	-
Fine diatomite earth	<LOD	-	-	<LOD	-	-
Cross-flow membrane	<LOD	-	-	<LOD	-	-
Flash-Pasteurisation	**1.4**	0.15	10.85	<LOD	-	-
Bentonite	0.14	0.02	18.74	<LOD	-	-
Silica gel	<LOD	-	-	<LOD	-	-
SensoVin - double dose in ppm				**After sterile EK**		
	Mean in ppm	SD	CV in %	Mean in ppm	SD in ppm	CV in %
Sterile filtration (K-100+EK)	<LOD	-	-	<LOD	-	-
Cartridge membrane 0.45 µm	<LOD	-	-	<LOD	-	-
Centrifuge	<LOD	-	-	<LOD	-	-
Fine diatomite earth	<LOD	-	-	<LOD	-	-
Flash-Pasteurisation	**1.4**	0.62	43.93		-	-
Bentonite	0.11	0.01	13.34	<LOD	-	-
Silica gel	**0.6**	0.35	61.5	<LOD	-	-

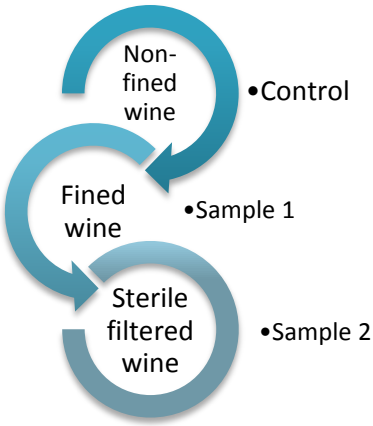
(Source: modified from AIF 16330 N, 2012)

5.1.2 Second trial wines (2011)

ELISA results for casein and ovalbumin from Hamburg

On the second trial all three white wines (white cuvée, Riesling and Mueller-Thurgau) fined with casein, at normal winemaking dosage (to be found on material and methods chapter) have a negative result for indirect ELISA, namely no casein residues have been detected in any of the samples. Three samples of each wine were analysed; control, fined without filtration and fined with an EK/sterile filtration.

Second trial schema



For the three red wines of the second trial (red cuvée, Pinot Noir and Sangiovese), fined with ovalbumin, one single wine has a positive result: Sangiovese (wine No. 8). But the positive result was the same for control wine, non-fined as well. This wine is the one with more "body", namely more phenolic compounds. According to responsible for ELISA in Hamburg, this might be a reading error caused by high phenol contend in the matrix wine. Therefore further developments and improvements were made for coming year of research.

ELISA through enzymatic kits of r-biopharm

The tests were done in the laboratory of r-biopharm AG, Darmstadt, Germany, on 27th of July 2011. The wines used are No. 3 to 10 from second trial.

Ovalbumin and Casein

On the second trial there are a greater number of wines, but the concentration applied here is lower and closer to reality than on the first trial, where worst-case scenario took place. Results are negative or under the limit of detection for all ovalbumin fined wines of Table 28 as well as for casein fined wines from
Table 29 all wines have been filtered with EK filter as a final step.

Table 28 ELISA of egg proteins (test kit). Red wines (No. 6 to 8) of trial 2

Wine variety and No.	Sample	Lysozyme [] applied	[ ] in ppm	Measure	Mean in ppm	Dilution
Cuvée No.6	Control	0	0	< LOD	< LOD	none
	Egg proteins	5g/hL	50	< LOD	0.118	none
Pinot Noir No.7	Control	0	0	< LOD	< LOD	none
	Egg proteins	5g/hL	50	< LOD	0.080	none
Sangiovese No.8	Control	0	0	< LOD	< LOD	none
	Egg proteins	20 g/hL	200	< LOD	0.075	none
Control solution	Solution with egg proteins	0.45 g/hL	4.5		3.81	

LOD: Limit of Detection

Table 29 ELISA of casein (test kit). White wines (No. 3 to 5) of trial 2

Wine variety and No.	Sample	Casein [ ] applied	[ ] in ppm	Measure	Mean in ppm	Dilution
Riesling No.3	Control	0		< LOD	< LOD	none
	Casein	10 g/hL	100	< LOD	0.181	none
Blend No.4	Control	0		< LOD	< LOD	none
	Casein	20 g/hL	200	< LOD	0.180	none
MT No. 5	Control	0		< LOD	< LOD	none
	Casein	20 g/hL	200	< LOD	0.177	none
Control solution	Solution with casein	0.8 g/hL	8		6.85	

LOD: Limit of Detection

Lysozyme

The results of this second trial on red wine show that readings are under the limit of detection on Table 30. The only presence of lysozyme with very high value is for the deposit of fining, after the fining itself, which was taken from the bottom of the fining recipient, with the intention to confirm the presence of lysozyme in the deposit. The Sangiovese red wine used for this trial has an elevated amount of phenols (Table 78) to react with lysozyme precipitating over 1200 ml from 25 litres of wine, as to be seen on Figure 73.

The precipitation of lysozyme and phenols could explain the negative values, even directly 24 hours after application. The high positive result of lysozyme on the deposit may be used as an explanation of the precipitation itself. Furthermore this effect is already well known and lysozyme application in high phenol containing wines is even contra-indicated by some producers of lysozyme (Martin Vialatte, 2013).

Table 30 ELISA of Lysozyme (test kit). Red wine Sangiovese (No. 10) of trial 2

Sample	Concentration applied g/hL	ppm	Measure ppm	Mean in ppm	Dilution
Control			< LOD	n.d	none
Lysozyme (L)	50	500	< LOD	0.015	none
L+ Bentonite (B)	50/200	500	< LOD	0.017	none
L+B +Metatartaric acid (Met)	50/200/10	500	< LOD	0.015	none
LB +Met (30°)	50/200/10	500	< LOD	0.019	none
LB +Met (30° - 17°)	50/200/10	500	< LOD	0.017	none
LB +Met -cold ELISA	50/200/10	500	< LOD	0.019	none
After addition deposit	50 g/hL	500	> 4000		

LOD: Limit of Detection **n.d.:** not detectable

As to be seen on Table 31 control wine, has no detectable allergen. Wine treated only with lysozyme has about the same amount as added (17 ppm over). This may be an error due to the strong required dilution and it should be taken into consideration that this value is out of the LOQ of the test kit.

Table 31 ELISA of Lysozyme (test kit) Riesling- White wine (No. 9) of trial 2

Sample	Concentration applied g/hL	ppm	Mean ppm	Dilution
Control			< LOD	none
Lysozyme (L)	50		> LOQ/517*	1:1250
L+ Bentonite (B)	50/350	500	147.46	1:1250
L+B +Metatartaric acid (Met)	50/350	500	< LOD	1:1250
LB +Met (30°)	50/350/10	500	< LOD	1:1250
LB +Met (30° - 17°)	50/350/10	500	< LOD	1:1250
LB +Met -cold ELISA	50/350/10	500	< LOD	1:1250
LB+Met deposit	50		> 4000	
Lysozyme solution	0.035	0.350	0.373	

*Result out of LOQ: Limit of Quantification. LOD: Limit of Detection

Wine treated with lysozyme and fined with bentonite has less than half of the added value, confirming once more what other studies have already showed before (Webber-Witt, 2009). Furthermore bentonite is well known to inactivate lysozyme since they have opposite charges and it is absorbed by bentonite.

All other samples are negative or under the limit of detection apart from the deposit. Deposit of fined wines has a high positive value. The high amount found in the deposit indicates that lysozyme has been precipitated by combination of fining with bentonite and further addition of metatartaric acid. Metatartaric acid precipitates and makes lysozyme insoluble from colloidal solution, as to be seen on picture Figure 74. The wine was clear and had no deposit after being treated with lysozyme. Further fining with bentonite and filtration with a "polish filtration" (K-100) left likewise a clear wine with no precipitation. Contrasting this clarity, the addition of 10 g/hl of metatartaric acid left a highly turbid and precipitated wine. Previous studies have appointed metatartaric acid and tannins as being responsible for cloudiness in wines and warning by lysozyme producers to avoid this combination is also not new (Martin Vialatte, 2013).

Even though precipitate sample "LBM deposit" of Table 31 has a great positive signal, which clearly indicates addition of metatartaric acid causes it to precipitate, the result for further samples treated with metatartaric acid were negative for lysozyme.
At this point of the work the questions on this respect have been posed;

- If lysozyme was being completely precipitated or if there was any kind of reaction with metatartaric acid maintaining lysozyme in solution and thus being detected.
- Metatartaric acid is unstable. Its stability is especially temperature dependent, as already mentioned on literature chapter of this work. As this acid deteriorate fairly rapid and loses its protective properties.

Therefore some wine samples were kept in warm cabinet long enough to completely hydrolyse metatartaric acid and check if lysozyme was at that point present. No positive signal was found in any of the samples; LB+Met (constant 30°C) nor LB+Met (alternation between 30°-17°).

Further precaution was taken during ELISA tests. During one of ELISA's step, samples are warmed up, therefore a double sample –one warmed and another not warmed (on purpose, since met. acid is temperature sensitive) have been prepared as to be seen on following table. However no positive result was found.

Further tests on "third trial" were done with another sample with no bentonite fining in between, for more precision. This may be seen on Table 32. Nevertheless authors mention that metatartaric acid may be removed by fining and that it could be unstable causing cloudiness (Moreno-Arribas *et al.*, 2009 – page 140).

5.1.3 Third trial wines (2012)

First part – Lysozyme, Results from Hamburg

On the third trial, repetition of previous trials with modifications, wines were treated with lysozyme, metatartaric acid, CMC and bentonite. Samples passed no filtration afterwards.

The results for all "control wines" or non-fined, for red and white (wine No. 11 to 16), as expected, have no detectable allergens, as well as wines treated only with metatartaric acid.

The quantity of lysozyme detected by ELISA after its addition is similar on the different wine varieties used. Added amount was 300 ppm. Wines treated with lysozyme shows values from 150 to 77 ppm, see Table 32. There is one exception showing a value of 8 ppm or 0.8g/L, which is the Cabernet Sauvignon Merlot wine (No. 14). Considering the amount of lysozyme added: 300 ppm there is a residue rate of around 50% for white wines and Pinot Noir, 25% for Dornfelder and only 3% for Cabernet Sauvignon Merlot.

An addition of metatartaric acid after lysozyme shows a lower reading of lysozyme on all wines. On Mueller-Thurgau wine the reduction of lysozyme has an importance of 74% and 64% for Riesling. Further fining with bentonite reduces the amount of lysozyme to not detectable readings. What is new and different on this third trial repetition is the use of lysozyme and metatartaric acid alone, without bentonite. All samples "lysozyme + metatartaric acid" the result is positive, meaning that this polymer used in a quantity of 10g/hL (highest legal value) is not capable to "remove" lysozyme itself, but only to reduce it to a lower value.

CMC addition (in 10g/hL) after lysozyme acts likewise reducing the amount of this protein in all wines, but to a lower degree than with metatartaric acid, to be seen on Table 32. The reduced quantity is lower than metatartaric acid though, for Mueller-Thurgau and Dornfelder the reduction of lysozyme is of 34% and for Pinot Noir 60%.

Again as in previous year the results of ELISA have a connection with wine deposit quantity. This time pictures of all samples have been put together to compare it with results and with each other as to be seen on Figure 76 and Figure 77. They are showing Mueller-Thurgau and Riesling wines, respectively.

- Control and metatartaric acid samples are clear and have negative signal for ELISA.
- Lysozyme sample has an extremely fine deposit, almost invisible, by ELISA there is a positive signal of 151 ppm for Mueller-Thurgau and 129 for Riesling.

- Lys+Meta sample have a deposit of around 35mL and big particles in solution after 1h and 15min and after 16h the deposit settles to half of it and there are still some particles in solution, but wine is less turbid than in the beginning. This precipitation meaning reduction, but still present in solution is confirmed by ELISA with 39 ppm for Mueller-Thurgau and 50 for Riesling.
- Lys+Bento+Meta have negative signal for both wines, here for the 4th time confirming previous studies
- Lys+CMC sample is extremely turbid after 1h 15min but also after 16h, showing a very minor deposit. ELISA results are of 101 ppm for Mueller-Thurgau and of 123 ppm for Riesling, again showing good correlation of picture and ELISA results.

On Figure 78 all red wines are together under each other after 2h 15min and as white wines these pictures can be well correlated to ELISA results. Sample number:

- Control is clear and has no deposit in all wines
- Lysozyme on Cab.Sav. wine has the greatest deposit of almost 100mL, the second is Dornfelder with almost 50mL and finally Pinot Noir with an unimportant deposit. These is directly correlated to ELISA results where Cab.Sav. has a reduction down to 8 ppm, Dornfelder down to 77 and Pinot Noir only down to 137 ppm. This is clearly explained by the phenol contend of wines, the higher the content the most dramatic the drop.
- Metatartaric acid shows no deposit in all samples.
- When lysozyme and metatartaric acid are used together there is a notable deposit in all three wines, they have all quite similar quantities, around 75mL for Cab.Sav. and over 50 mL for Pinot Noir and Dornfelder. ELISA results are similar and follow Lysozyme sample trend: Cab.Sav. has the greatest reduction and Pinot Noir the lowest, but values here are closer: 6 ppm and 20 respectively.
- Samples treated with lysozyme then bentonite and finally with metatartaric acid have one of the major deposits, again for Cab.Sav. one can observe the greatest of around 135 mL and other wine just over 120 mL. Results are equal – in none of the wines a positive. Meaning that all lysozyme is removed i.e. cannot be detected.
- 6 Deposits here were around 200mL for Cab.Sav (to be seen on the picture there was floating particles on the top of the cylinder), around 80 mL for Pinot Noir and a bit over 50mL for Dornfelder. Results are comparable for ELISA where Cab.Sav is reduced to 4 ppm, Dornfelder to 51 ppm and Pinot Noir to 56 ppm.

The interest of finding out the interaction of metatartaric acid and lysozyme could be well observed on this experiment. It is possible to conclude that in wines with high phenol content, especially in reds; metatartaric acid alone is capable of reducing to not detectable the amount of lysozyme. In white wines it only happened when bentonite was added.

Table 32 ELISA-Hamburg - lysozyme residues measurements of third trial

Wine/Variety	Sample/Treatment [ppm]	Lysozyme reading (ppm)	CV (%)
Mueller-Thurgau Wine No. 12	Control	n.d.	
	Metatartaric acid	n.d.	
	Lysozyme [300]	150.8	6.3
	Lysozyme+Metatartaric acid	39.2	5.1
	Lysozyme+Bentonite+Metatartaric acid	n.d.	
	Lysozyme+CMC	100,7	6.4
Riesling Wine No. 13	Control	n.d.	
	Metatartaric acid	n.d.	
	Lysozyme [300]	128.5	6.4
	Lysozyme+Metatartaric acid	50.0	3
	Lysozyme+Bentonite+Metatartaric acid	n.d.	
	Lysozyme+CMC	122.8	6.1
Cab. Sauv/Merlot Wine No. 14	Control	n.d.	
	Metatartaric acid	n.d.	
	Lysozyme [300]	7.9	6
	Lysozyme+Metatartaric acid	5.6	3.1
	Lysozyme+Bentonite+Metatartaric acid	n.d.	
	Lysozyme+CMC	4.4	0.7
Dornfelder Wine No. 15	Control	n.d.	
	Metatartaric acid	n.d.	
	Lysozyme [300]	77.2	0.8
	Lysozyme+Metatartaric acid	8.7	3.6
	Lysozyme+Bentonite+Metatartaric acid	n.d.	
	Lysozyme+CMC	51.1	6.5
Pinot Noir Wine No. 16	Control	n.d.	
	Metatartaric acid	n.d.	
	Lysozyme [300]	136.8	2.7
	Lysozyme+Metatartaric acid	19.5	9.3
	Lysozyme+Bentonite+Metatartaric acid	n.d.	
	Lysozyme+CMC	55.8	0.9

n.d: not detectable (Source: modified from AIF 16330 N, 2012)

Second part – casein and ovalbumin

Following table shows the results of wines fined with casein and/or ovalbumin and not filtered.

Only one white wine shows positive result; it is a Riesling fined with casein and the detected amount is of 0.23 ppm. This value is positive, but according to law do not need to be declared on the label (Christmann *et al.,* 2012). While the same in untrue for red wines fined with ovalbumin, all wines have a positive result, varying from 1.5 to 0.47 ppm. Results for ovalbumin may lead to make winemakers aware of fining with egg white/ovalbumin and not filtering the wines lately to have wines checked or filtered before bottling, to be sure about label declaration. Also to be observed on Table 33 is the white wine fined with ovalbumin (100 ppm) which has no allergens found or a result lower of detection limit. This result was different when 160 ppm was used on first year trial on this study, as to be seen on Table 25 where a Müller-Thurgau wine of different year has in all wines detectable residues when not filtered.

Table 33 ELISA Hamburg Casein & ovalbumin on non-filtered wines -3rd trial

Variety	**Casein added [ppm]**	**Casein reading [ppb]**	**[ppm]**	**CV%**
Chardonnay Wine No. 11	100	<LOD	<LOD	
Mueller-Thurgau Wine No. 12	100	<LOD	<LOD	
Riesling Wine No. 13	100	231.5	0.23	8.2
Variety	**Ovalbumin added [ppm]**	**Ovalbumin reading [ppb]**	**[ppm]**	**CV%**
Cab. Sav. Merlot Wine No 14	100	1508.7	1.5	6.4
Dornfelder Wine No 15	100	706.2	0.7	10.2
Pinot Noir Wine No 16	100	467.6	0.47	7.0
Mueller-Thurgau Wine No. 12	100	<LOD	<LOD	

CV: coefficient of Variation; LOD Limit of Detection

Third part (ELISA results from Hamburg)

Flash pasteurisation and centrifugation (second repetition)

As above mentioned this trial is a repetition to confirm previous positive results for flash-pasteurisation and centrifugation.

The positive value of 0.32 ppm for centrifuged white wines on Table 34 may be explained though particle size and centrifuge mechanisms. Physical forces weren't strong enough to separate the proteins that are in colloidal solution. The electric charge and solubility are also factors in the stability of colloidal particles in wine. The status of the electric charge is dependent mainly on pH. Flocculation are induced only when electric charges are neutralised, or at its isoelectric point -pI (Farbas, 1988). Nevertheless after a sterile filtration particles are removed and ELISA has a negative signal.

As for flash pasteurisation, different of previous year where there was an elevated allergen content after flash-pasteurisation, in this trial-repetition no particle can be found before neither after pasteurisation.

Table 34 ELISA Results for Casein Flash pasteurisation and centrifugation (2nd repetition)

White wine –Mueller-Thurgau	reading of fining agent (ppm)	CV (%)
Control wine (not filtered neither fined)	<LOD	
Control wine after Flash-Pasteurisation (FP)	<LOD	
Casein fined wine (400 ppm) with no further treatment	<LOD	
Casein fined wine (400 ppm) after FP	<LOD	
Casein fined wine (400 ppm) after FP +EK* filtration	<LOD	
Casein fined wine (400 ppm) after centrifugation	**0.32**	8.18
Casein fined wine (400 ppm) after centrifugation +EK Filtration	<LOD	

LOD Limit of Detection; *EK Sterile filtration

On this repetition of Table 35, results are negative for samples directly after fining and after flash-pasteurisation, but positive after EK-filtration. This result is peculiar and might be subject of a possible reading error. There are no residues already after the fining, where no filtration was carried out. Furthermore these results were presented by the project partner after project was concluded. Therefore this wine won't be taken into consideration on conclusion chapter.

Table 35 ELISA Results for Ovalbumin Flash pasteurisation and centrifugation (2nd repetition)

Red wine –Cuvée (80% PN)	reading of fining agent (ppm)	CV (%)
Control wine (not filtered neither fined)	<LOD	
Control wine after Flash-Pasteurisation (FP)	<LOD	
"AlbuVin" fined wine (160 ppm) with no further treatment	<LOD	
"AlbuVin" fined wine (160 ppm)after FP	<LOD	
"AlbuVin" fined wine (160 ppm)after FP +EK* filtration	LOD<x<LLOQ	
"AlbuVin" fined wine (160 ppm) after centrifugation	**0.71**	10.20
"AlbuVin" fined wine (160 ppm) after centrifugation +EK Filtration	**0.39**	31.03

^Measuring range; *EK Sterile filtration; LOD Limit of Detection; LLOQ Lower Limit of Quantification

5.2 Clinical trials

The laboratory of the Department of Dermatology and Allergology in the Technical University of Munich was in charge of allergic patients' recruitment for in vivo test of wines presenting potentially allergenic residues of fining agents from chicken egg and/or cow's milk, which was the final step for this study. It is important to mention that it is extremely challenging to recruit patients for allergy testing, maybe being one of the leading obstacles on allergy studies. The Department has a database of the preceding five years before the beginning of this study, in which food allergy was detected and tested with positive serum IgE assay. Out of 16,461 patients, 1,931 were children and adolescents (0-18 years), and 14,530 were adults. From those only 331 were positive IgE for egg or milk, and from them only 161 were allergic to cow's milk. Of these 331 patients, only 32 adult patients suffered a clinically evident food allergy to egg and/or milk (only one person presented both an allergy to hen's egg and cow's milk). Finally, only 10 patients, from the 32 were included in the test, due to lack of time, pregnancy, strict alcohol abstinence, among others. Each one from these 10 reported a positive anamnesis after ingestion of chicken egg or cow's milk, as well as for skin prick test. The patients were eight women and two men ranging from 26-63 years of age (mean age 42). The recruitment diagram can be seen at annexes chapter.

Prick skin test (PST) and double-blind, placebo-controlled food challenge (DBPCFC) trial were performed to determine whether any allergic reactions followed consumption would be trigged by wines fined with casein. The wines were a white and a red wine fined with 400 mg/L Kal-Casin Leicht löslich® after being sterile filtered, or sample 3 of Figure 1. Blood samples were taken to detect sensitization to food allergens, and to test the allergenicity of fined wine (30mL whole blood in adults). For the PST to be

considered a positive result in this study a wheal has to have a diameter greater than 3 mm with accompanying erythema greater than 5 mm compared to the negative control. The positive control was carried out with 0.1% histamine and the negative control containing 0.9% NaCl.
The oral provocation with fined (verum) and no fined (placebo) wine was conducted on two days, the time interval between treatment and placebo testing was at least 48 hours. It takes place in two "test blocks" for each one day. On the first day patients ingest the wine to be tested in an ascending amount, starting with a single drop, 0.1 mL, after 30 minutes a very small volume of 1 mL, if no symptoms are felt, after 60 minutes, a larger amount of wine is given (10 mL). If after another 30 minutes no symptoms occur, then the remaining 189 mL of wine is given for women and the remaining 289 mL for men. Thus, the total dose was 200 ml in women and 300 ml in men. In case of possible responses, the patient was in observation for roughly 2 more hours. On the second day patients repeated the procedure, but with another wine.

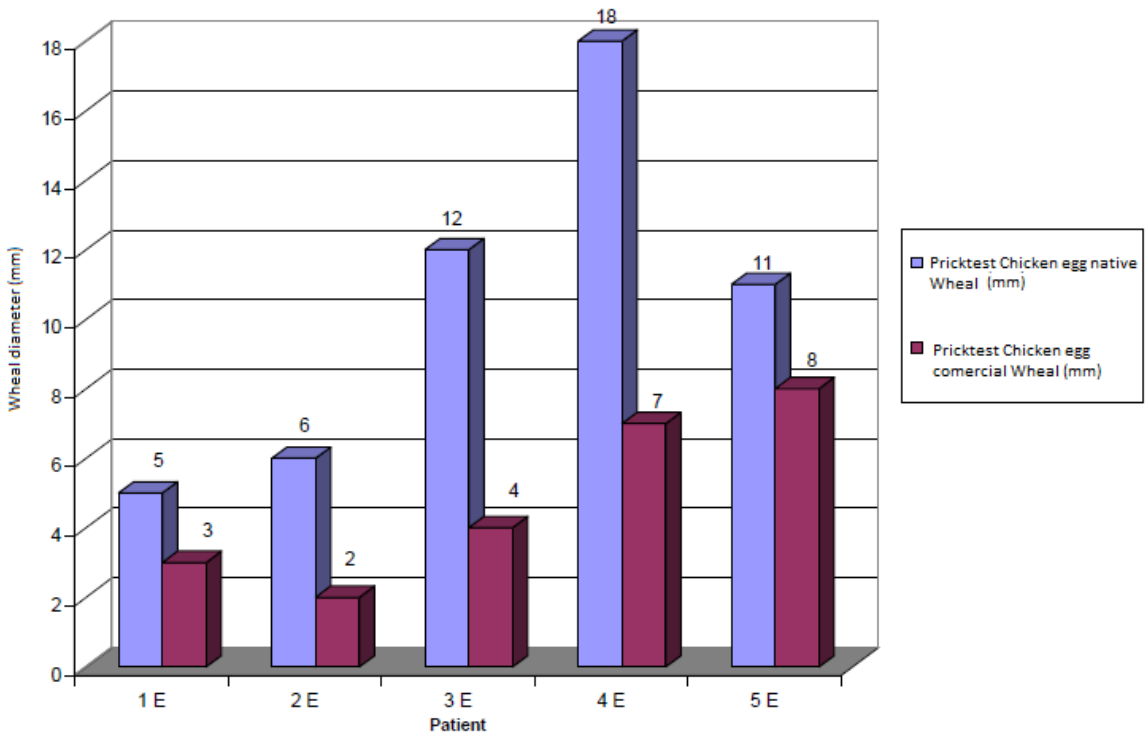
The tests have been done with wines fined in Geisenheim, as well with the same fining agents used in the wines, then analysed by ELISA in Hamburg and after ELISA results were prompt present and showed results safe enough, subjects were tested. The ELISA measure is the *in vitro* part of project and these results are the *in vivo* part.

5.2.1 Skin prick test

This immune-study considers a result positive when a wheal has a diameter greater than 3mm with accompanying erythema greater than 5mm compared to the negative control. The positive control was carried out with 0.1% histamine and the negative control containing 0.9% NaCl. Meaning that each patient has a different number to indicate it is positive or negative. Therefore all bars charts are described below.

Allergic patients to egg showed strong reactions to native pasteurized egg in commercial solution: Figure 34. This test confirms their allergy to egg.

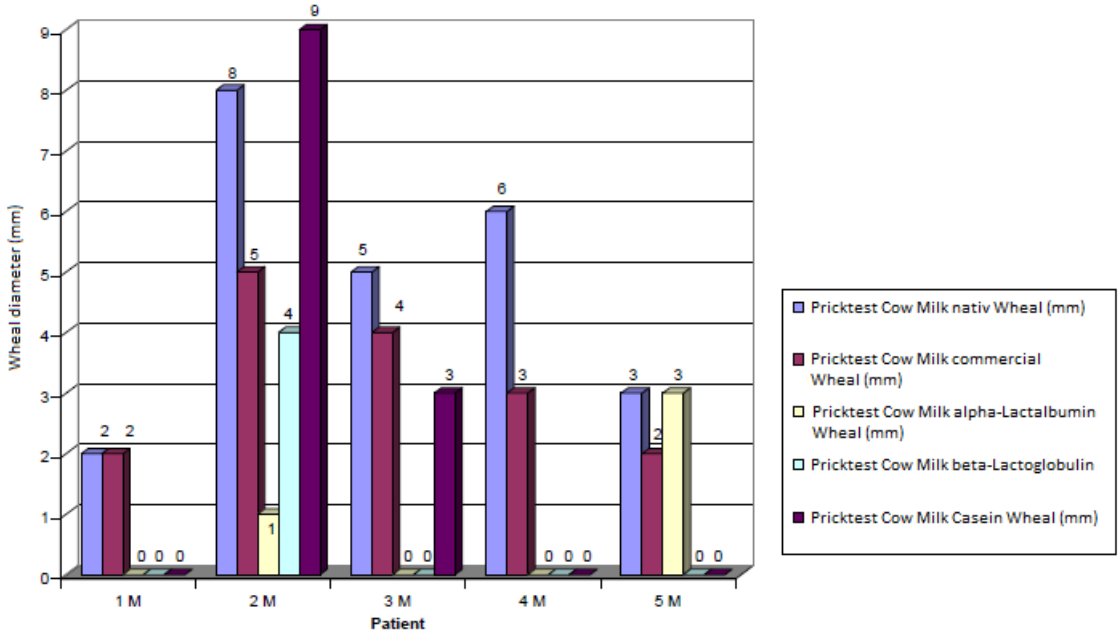
Figure 34 Bars chart of Prick test results – native & commercial chicken egg



(Source: modified from AIF 16330 N, 2012)

All patients allergic to milk (n = 5) show a positive reaction to native cow milk and only three to commercial milk extract Figure 35. Two patients reacted positive to casein. One patient reacted to β-lactoglobulin and another to α-lactalbumin.

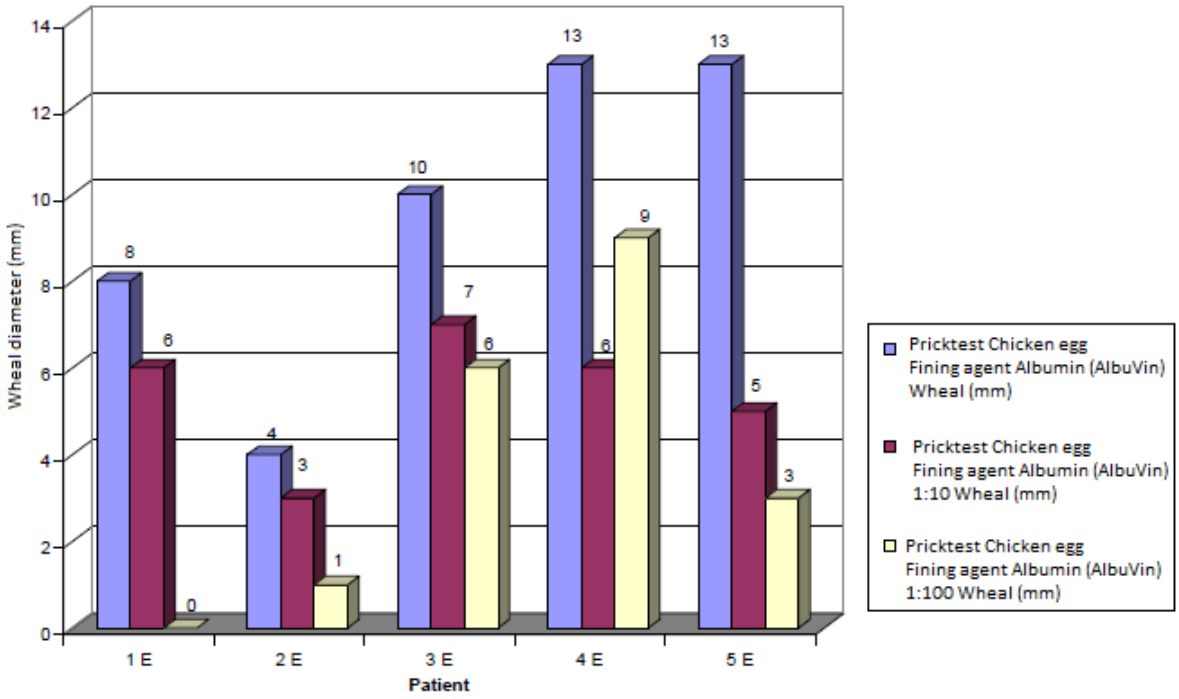
Figure 35 Bars chart of Prick test results - native milk and others milk proteins



(Source: modified from AIF 16330 N, 2012)

Patients have been tested with AlbuVin, chicken egg fining agent. All patients showed positive reaction for non-diluted AlbuVin, as well as for dilution 1:10. For concentration 1:100 there were three patients with positive reaction, Figure 36.

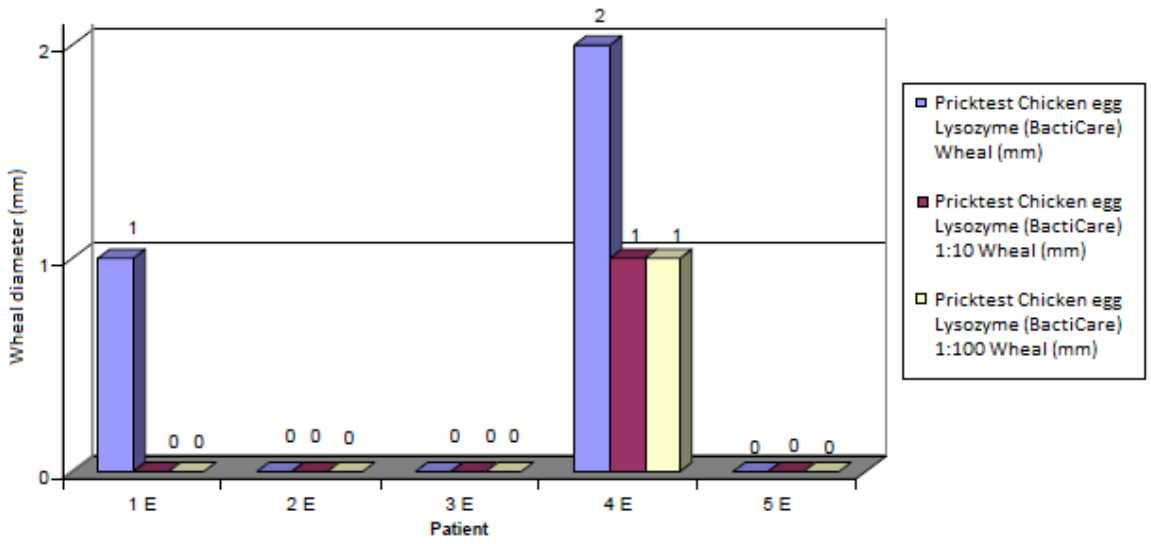
Figure 36 Bars chart of Prick test results – AlbuVin, egg fining agent



(Source: modified from AIF 16330 N, 2012)

The results are for lysozyme agent BactCare in all patients negative Figure 37.

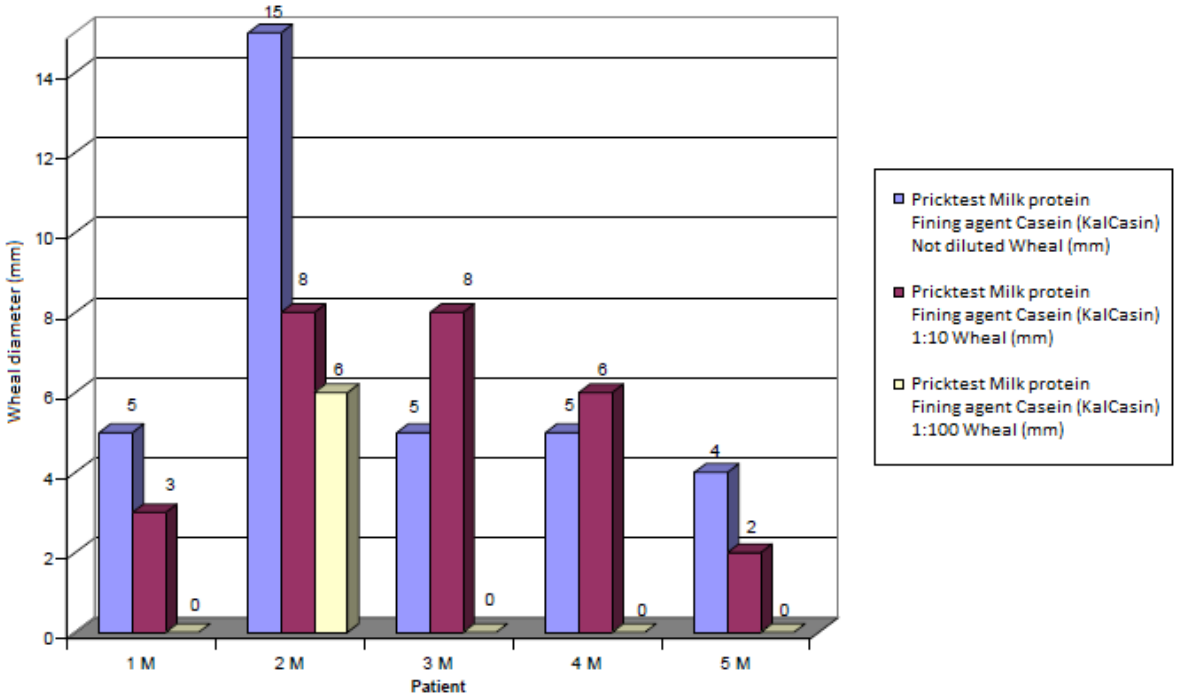
Figure 37 Bars chart of Prick test results - BactCare Lysozyme



(Source: modified from AIF 16330 N, 2012)

Casein fining agent Kal-Casin has a positive reaction on all patients when not diluted, in four patients when dilution is 1:10 and only one patient showed reaction to concentration 1:100 Figure 38.

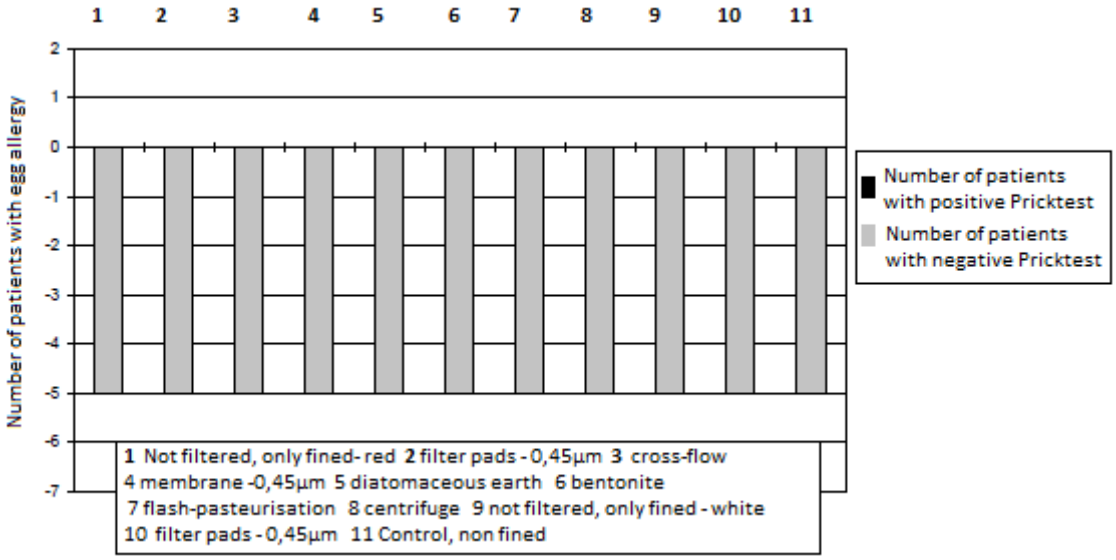
Figure 38 Bars chart of Prick test results - Kal-Casin, Casein fining agent



(Source: modified from AIF 16330 N, 2012)

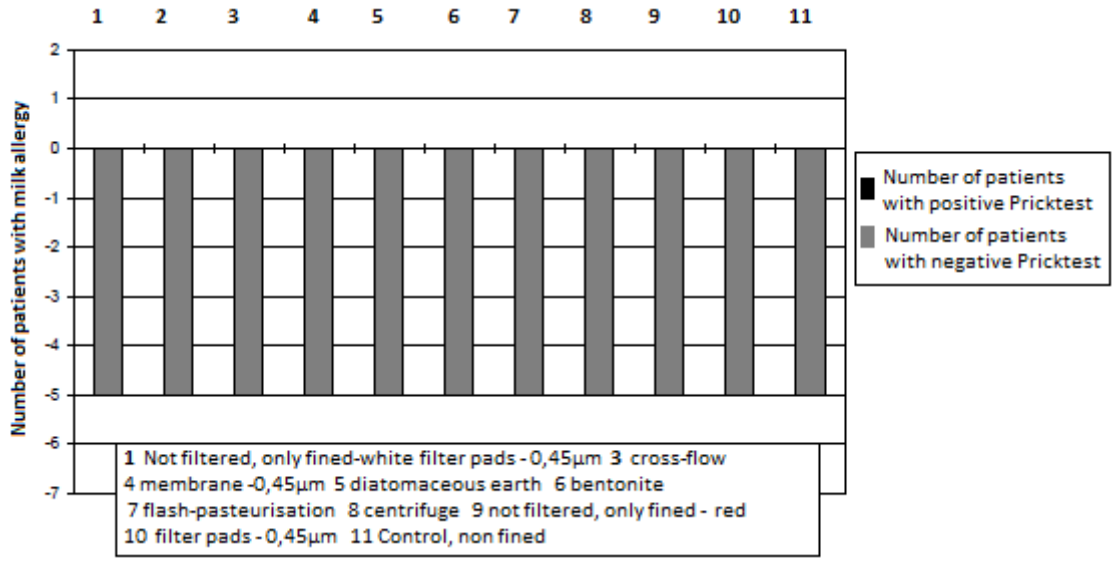
A prick test was done for every different method of filtration and separation used on the first trial of this work; for AlbuVin, with egg allergic patients and with Kal-Casin for casein allergic patients. Both tests with red and white wines (wine No. 1 and 2). No positive result has been found as to be confirmed on following figures.

Figure 39 Bars chart of Prick test results for egg- all separation methods



(Source: modified from AIF 16330 N, 2012)

Figure 40 Bars chart of Prick test results for milk- all separation methods



(Source: modified from AIF 16330 N, 2012)

5.2.2 Double-Blind Placebo-Controlled Food Challenge - DBPCFC

For this double-blind placebo-controlled food challenge test all wine bottles have been properly blinded. The oral provocation with fined (verum) and not fined (placebo) wine was conducted in two days, the time interval between treatment and placebo testing was at least 48 hours.

For the *verum* testing on patient with egg allergy, the selected wine was the red variety Regent (wine No. 2) fined with ovalbumin, which passed through fine and sterile filtration. No residue of fining agent has been found in this wine after ELISA test results from Hamburg (see annexes chapter wine code 09936 EK Filtration A3 in AIF 16330 N, 2012). Placebo wine is the same wine without fining agent, which passed the same filtration as *verum*.
A white wine of Mueller-Thurgau (wine No. 1) fined with casein was selected as the *verum* for the provocation test with milk allergy patients. Also this wine has two filtrations as wine mentioned above, and here as well no allergen has been found (see annexes wine code 09915 EK Filtration K3 in AIF 16330 N, 2012). Placebo wine is the same wine without fining agent, which passed the same filtration as *verum*.

In a second run of the test, patients with egg allergy have been tested with a white wine treated with lysozyme, then with bentonite and filtered as both above mentioned wines (see annexes wine code 09918 EK Filtration L3 in AIF 16330 N, 2012).

Ten patients were challenged with the respective fined and not fined wines in the DBPCFC.

A patient with cow's milk allergy reported after 25 minutes next to the third stage of oral provocation tests and a cumulative amount of 11 ml *verum* wine, a slight "tingling" in the area of the left shoulder blade. This was seen as subjective symptoms. The oral provocation test was continued and finished without any problems arisen or further symptoms. Finally, since 200 ml were tolerated without any other symptoms, the response was classified as negative. The tingling formed spontaneously after about 40 minutes back.
None of the ten patients who were orally provoked showed a reaction in the sense of immediate allergic reaction, neither to the fined nor to the not fined wine. All patients tolerated the respective total dose of 200 mL (women) or 300 ml (men) of the verum and control wine.
Also for two patients allergic to egg, on the second run with lysozyme and bentonite, no reaction in the sense of immediate allergic reaction have been showed.

5.3 Phenols

5.3.1 Total phenols

Because the wine phenolics are the main wine substances affected by fining this study closes up on the main wine phenols. The intention is to look for possible further relation with fining reduction and residues.

Table 36 has the result for total phenols analysed with Folin-reagent (FCR) and by HPLC on wines 1 and 2 of project 1. The results for FCR are only given in catechin.

These analyses were done in January 2011. Values are the mean of N:2.

For some wines the result values are slightly higher than the control wine. A possible explanation is that the protein may disturb the readings. The highest reduction on phenols is for PVPP. Insoluble, PVPP, is only able to contact relatively few hydroxyls of larger molecules. Fining agent such PVPP binds with smaller phenols which conform to the PVPP particle. It is specific for small-molecular-weight phenols such as catechins (Zoecklein, 1990). Therefore it explains the difference comparing to others fining agents PVPP is the one that shows a greater reduction.

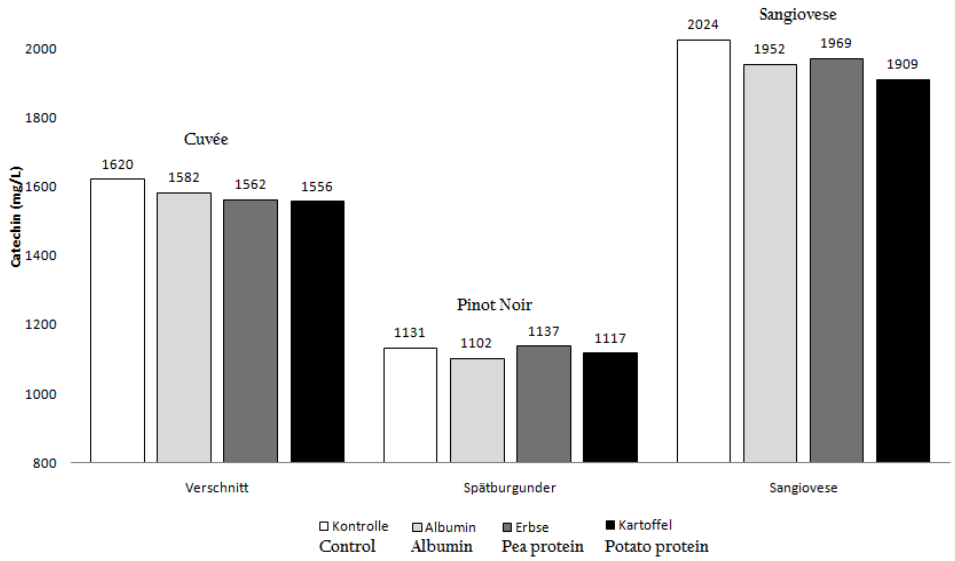
Table 36 Total phenols FCR (catechin) and HPLC

	FCR (mg/L)	**FCR (mg/L)**	**HPLC (mg/L)**
	MT/Wine1	Regent/Wine2	Regent/Wine2
Control	167	1240	365.2
Isinglass	178	1278	368.8
Ovalbumin	190	1241	368.6
Pea protein	171	1204	370.7
Gelatine	175	1231	372.2
PVPP	**154**	**1072**	**333.4**
Potato protein	189	1207	372.7
SensoVin	167	1203	374.4
Lysozyme	184	1205	377.4
Whey protein	183	1239	370.7
Casein	181	1240	364.4

The following figure shows the bar chart of total phenols (catechin mg/L) in wines No. 6-8 of second trial, by Folin-reagent (FCR).
Sangiovese has the highest phenol content followed by cuvée wine and the Pinot Noir has the lowest phenol content. For cuvée wine and for Sangiovese, the potato protein

shows the greatest reduction. Nevertheless all fining agents show slightly reductions compared to control.

Figure 41 Bars chart of total phenols (catechin mg/L) in wines No. 6-8

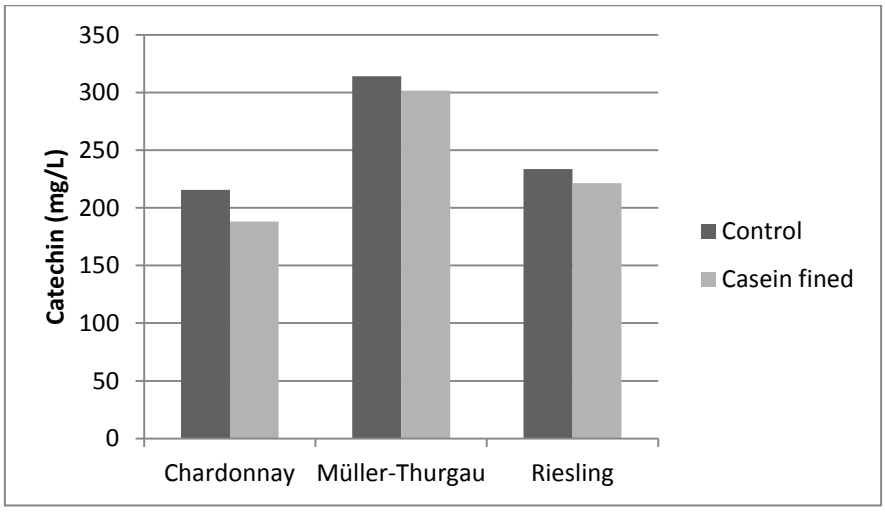


(Source: modified from Lesa – work done together, bachelor student)

The following figures show the bar chart of total phenols (catechin mg/L) in wines No. 11-16 of third trial, by Folin-reagent (FCR).

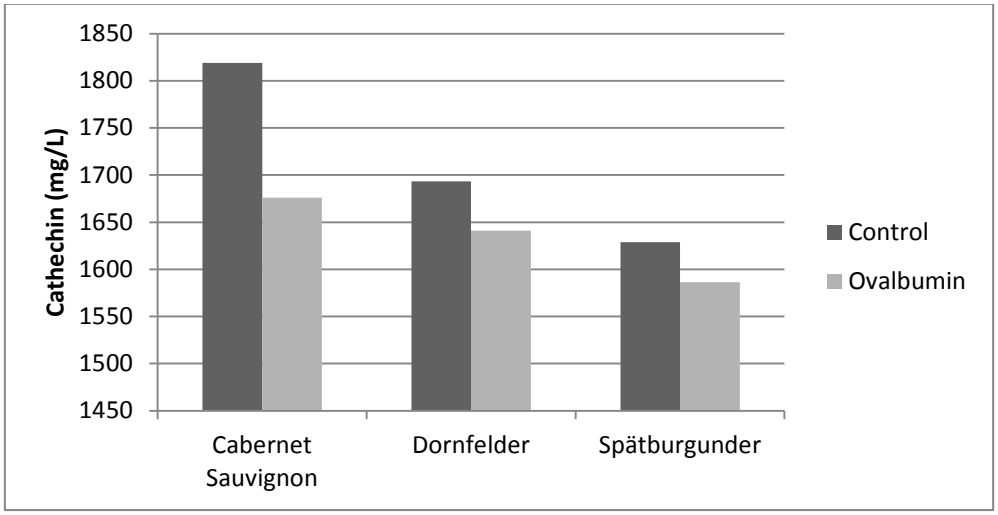
All fined wines show a reduction on the phenol content compared to control, showing that fining agents reacted with wine phenols. Casein is well known to reduce monomeric, oligomeric and polymeric flavanols from white wines as showed in other studies (Cosme *et al*, 2007)

Figure 42 Bars chart of total phenols (catechin mg/L) in wines No. 11 to 13



(Mean N:2)

Figure 43 Bars chart of total phenols (catechin mg/L) in wines No. 14 to 16



(Mean N:2)

5.3.2 Colourless phenols

For these analyse a white wine Mueller-Thurgau and a red wine Regent wine No. 1 and wine No. 2 have been used. The process and fining agents are described on first trial of material and methods chapter.

Table 37 Colourless phenols from Mueller-Thurgau - Wine No. 1

Fining agent	Tyrosol	Caftaric-acid	P-coumaryl-glycosil-tartaric acid	Coutaric-acid	Fetaric-acid	Coumaric-acid	Total sum of colourless phenols
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Control	18.4	8.9	0.9	1.4	1	1	**31.6**
Isinglass	16.3	8.8	1	1.4	0.9	1.3	**29.7**
Ovalbumin	17.6	9	1	1.4	0.8	1.1	**30.9**
Pea protein	17.1	8.5	0.8	1.4	0.9	0.9	**29.6**
Gelatine	17.8	8.8	0.9	1.5	0.8	1	**30.8**
PVPP	18	8.2	0.8	1.5	0.9	1.1	**30.5**
Potato protein	16.8	8.5	0.8	1.4	0.8	1.1	**29.4**
SensoVin	16.5	8.7	0.7	1.9	0.7	0.9	**29.4**
Lysozyme	16.8	8.7	0.8	1.8	0.8	1	**29.9**
Whey protein	15.6	8.7	0.8	1.9	0.6	1.2	**28.8**
Casein	18.5	8.4	1	1.4	0.9	1.4	**31.6**

Apart from casein all fining agents have diminished the total sum of colourless phenols on white wine of Table 37. Casein and ovalbumin are the proteins that reduce less, 0 and 2.2%, while whey protein is more reactive reducing 9%. This can be seen on the following bar charts.

Figure 44 Bar chart of colourless phenols Mueller-Thurgau wine

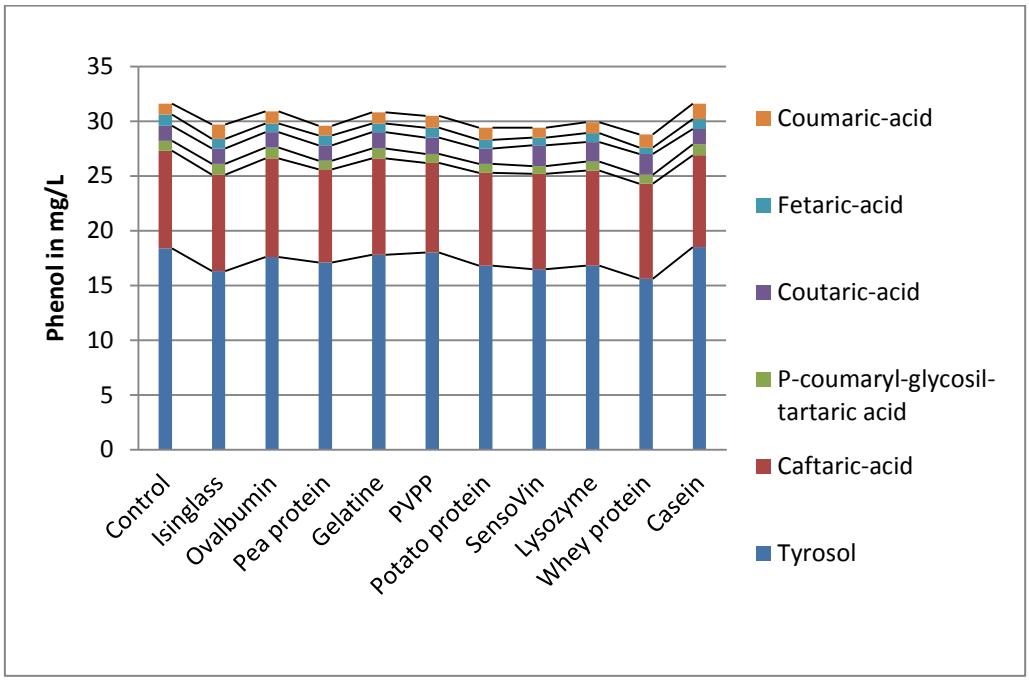


Figure 45 Bar chart of colourless phenols in %

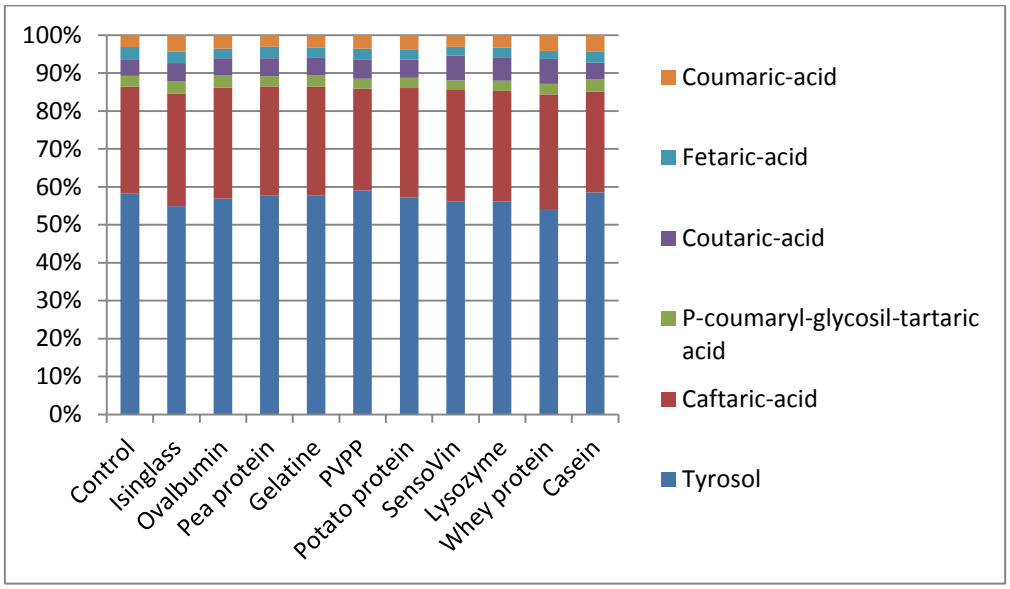


Table 38 Colourless phenols Regent - Wine 2

Fining agent/ Sample	Protocatechic acid	Procyanidin B1	Procyanidin B2	Tyrosol	Catechin	Epicatechin
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Control	3.9	9.7	11.4	20.9	29.2	8.8
Isinglass	3.8	9.0	11.5	20.15	29.8	8.1
Ovalbumin	3.5	10.8	11.4	20.7	30.2	8.2
Pea protein	3.5	10.6	11.9	21.2	30.0	8.6
Gelatine	3.3	9.4	12.0	21.3	28.2	8.4
PVPP	3.8	3.7	6.2	21.9	15.9	7.9
Potato protein	3.7	10.6	11.0	22.6	29.0	8.6
SensoVin	3.4	9.5	10.6	24.1	28.1	8.3
Lysozyme	3.5	10.3	11.2	25.3	28.7	8.3
Whey protein	3.2	10.7	11	22.5	29.3	8.1
Casein	3.9	10.2	11.3	21.2	29.6	8.3

There is a reduction on procyanidin B1 and B2 and catechin for PVPP in Table 38. PVPP can effectively adsorb phenols from wine (Laborde *et al.*, 2006).

Figure 46 Bar chart of colourless phenols Regent

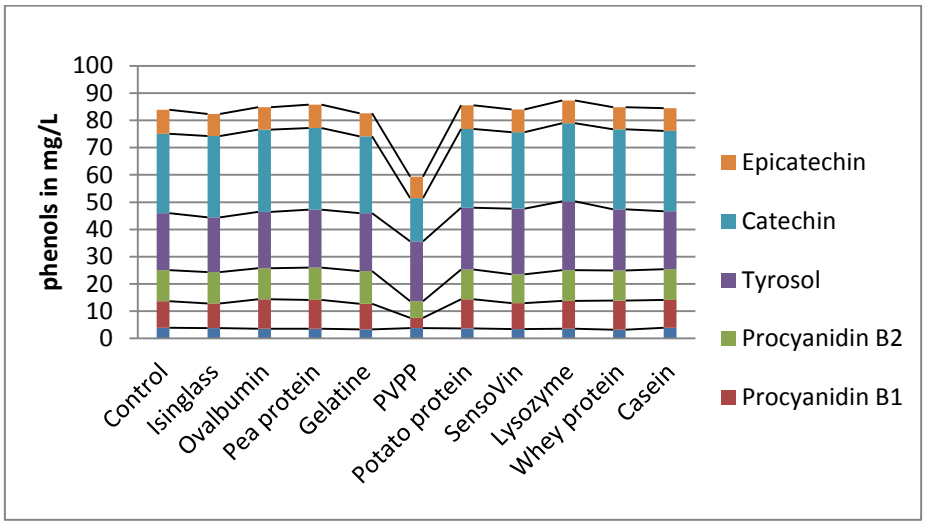


Figure 47 Bar chart of colourless phenols % Regent

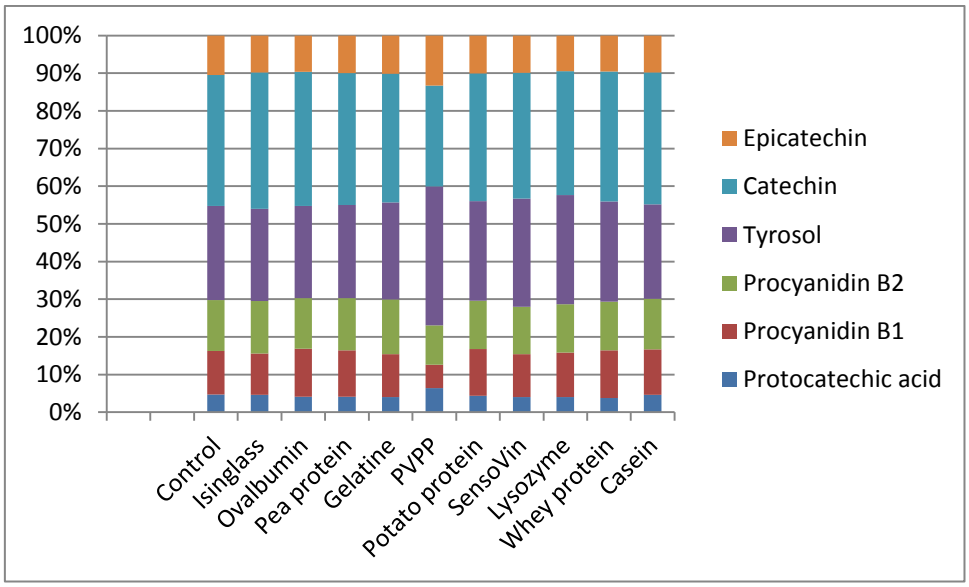


Table 39 Colourless phenols part II Regent - Wine 2

Fining agent/ Sample	**Caftaric-acid**	**P-coumaryl-glycosil-tartaric acid (p-CGT)**	**Coutaric-acid**	**Coumaryl - derivate**	**Coumaryl - derivate 2**	**Total sum of colourless phenols**
	mg/L	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Control	22.3	0.8	5.9	1.2	8.2	**122.3**
Isinglass	22.6	0.9	5.1	1.2	8.0	**120.2**
Ovalbumin	22.0	1	4.8	1.3	8.2	**122.1**
Pea protein	22.1	0.6	5.5	1.2	8.1	**123.3**
Gelatine	22.9	0.9	5.4	1.2	8.2	**121.2**
PVPP	21.3	0.9	5.6	1.2	7.3	**95.7**
Potato protein	22.4	0.9	5.0	1.3	8.1	**123.2**
SensoVin	22.1	1	5.3	1.3	8	**121.7**
Lysozyme	21.8	1	5.4	1.2	8.1	**124.8**
Whey protein	23.5	1	5.2	1.2	7.9	**123.6**
Casein	22.7	0.7	5.4	1.2	8.5	**123.0**

A stronger reduction on the sum of total colourless phenols can be found for PVPP (22%) on Table 39. PVPP reduces wine oxidation through elimination of polyphenols in reduced or oxidised forms that which comprises simple phenolic acids and flavonoids (Cosme *et al.*, 2011; Donel *et al.*, 1993).

PVPP eliminate tannins, oxidisable cinnamic acids and quinones formed when they oxidise. PVPP reacts specifically with low polyphenols such as monomers and dimers,

e.g. catechin and anthocyanin. Its binding action on leucoanthocyanins, catechins, flavonols and phenolic acids, take place between PVPP carbonyl and the phenolic hydroxyl. As the PVPP is insoluble, the phenolic molecule adsorbs on its surface and precipitates out of the solution (Rankine, 2004; Ribéreau-Gayon *et al.*, 2006; Jakob *et al.*, 1997; Margalit, 2004; Würdig and Woller, 1989 and Troost, 1988).

Figure 48 Bar chart of colourless phenols Regent II

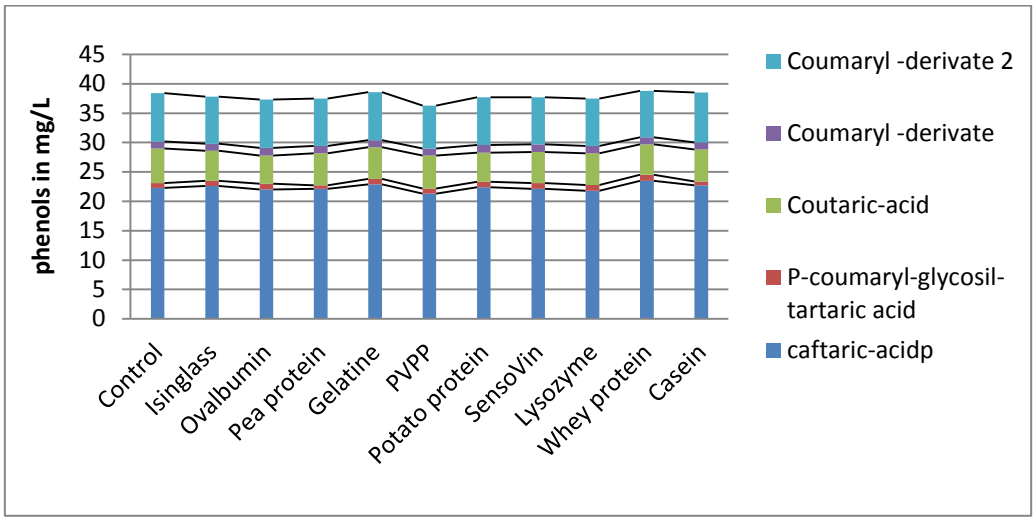
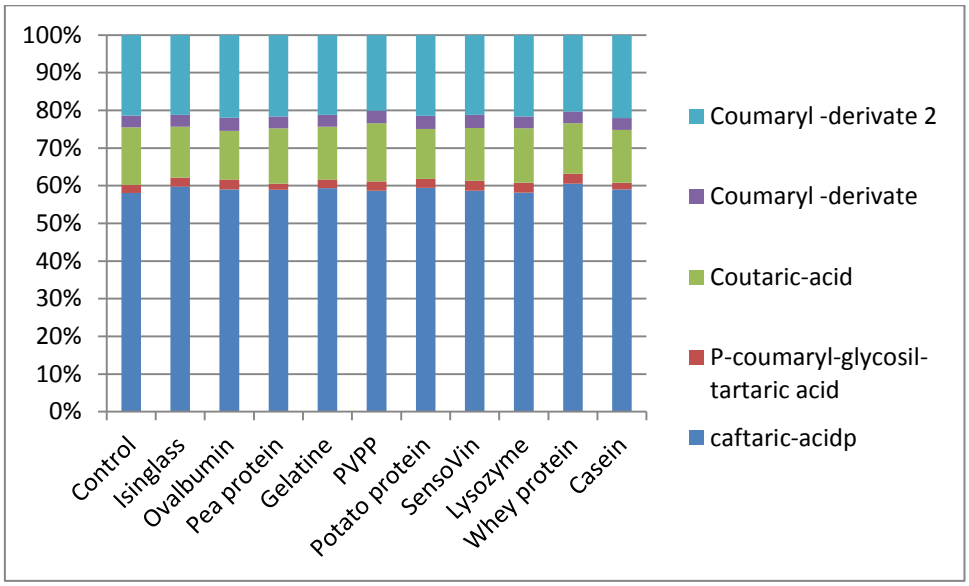


Figure 49 Bar chart of colourless phenols Regent II



5.3.3 Anthocyanins

These analyses have been done with wine No. 2, Regent. The process and fining agents are described on first trial of material and methods chapter. The following tables show

a detailed result of anthocyanins as well as its chromatogram for the main three allergens relevant for this work; ovalbumin, casein and lysozyme.

Table 40 Anthocyanins Regent -wine 2

Fining agent/ Sample	**Del-3,5-diglc***	**Cya-3,5-diglc***	**Pet-3,5-diglc***	**Del-3-glc***	**Peo-3,5-diglc***	**Mal-3,5-diglc***
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Control	10.2	6.0	18.7	9.3	32.7	157.7
Isinglass	10.1	6.0	18.7	9.3	32.1	155.4
Ovalbumin	10.2	6.0	18.8	9.3	32.4	156.3
Pea protein	10.1	6.0	18.7	9.3	32.8	159.5
Gelatine	10.1	6.0	18.8	9.4	32.3	155.6
PVPP	10.0	5.9	18.5	9.0	32.4	156.4
Potato protein	10.2	5.9	18.4	8.8	30.8	153.2
SensoVin	10.2	6.0	18.8	9.3	33.0	158.9
Lysozyme	10.2	6.0	18.8	9.4	32.9	159.2
Whey protein	10.1	5.9	18.8	9.3	32.8	158.2
Casein	10.0	4.9	17.3	8.8	31.6	153.6

Apart from casein only slightly difference are found on Table 40.

Figure 50 Bar chart of Anthocyanins Regent part I

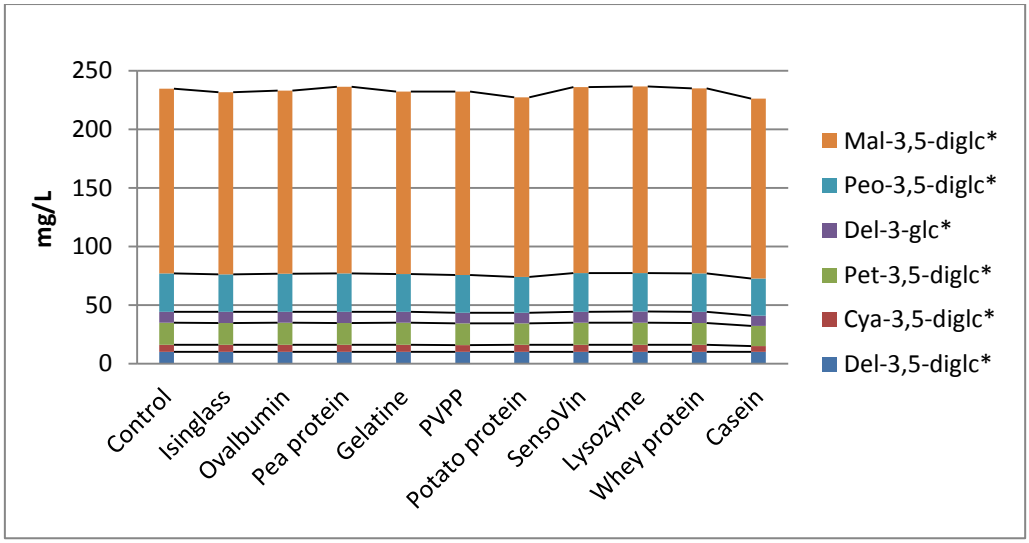


Figure 51 Bar chart of Anthocyanins Regent in % part I

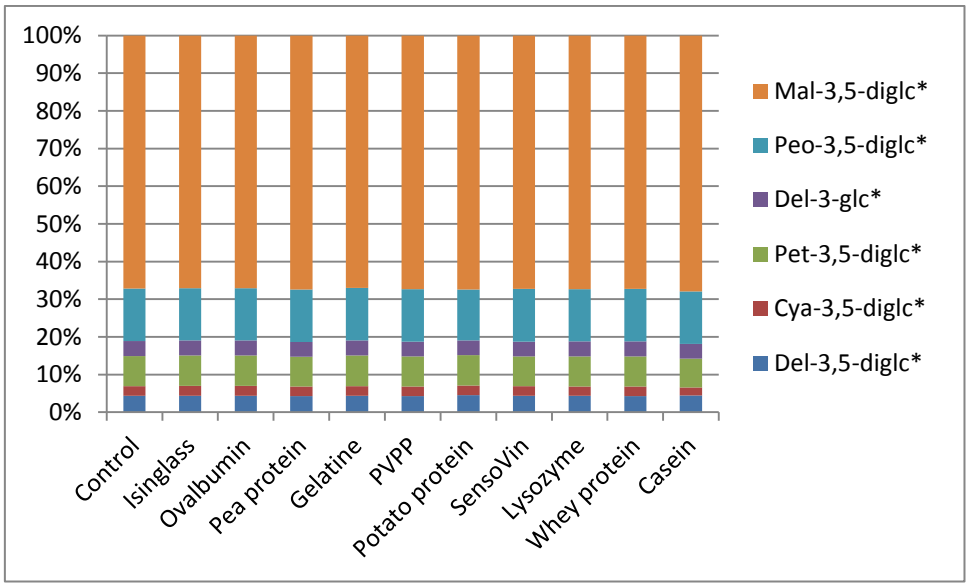


Table 41 Anthocyanins part II Regent - Wine 2

Fining agent/ Sample	**Pet-3-glc***	**Peo-3-glc***	**Mal-3-glc***	**Mal-3-glac***	**Pet-3.5-diglcu***	**Del-3-glcu***
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Control	14.1	3.2	49.6	5.6	2.8	3.9
Isinglass	12.8	2.3	49.8	5.6	2.8	3.6
Ovalbumin	13.0	2.5	50.2	5.6	2.8	3.6
Pea protein	12.9	3.2	49.9	5.6	2.8	3.5
Gelatine	13.0	3.2	50.1	5.6	2.7	3.7
PVPP	13.8	3.1	49.5	5.4	2.7	3.0
Potato protein	13.0	3.2	50.5	5.6	3.0	3.5
SensoVin	14.1	3.2	49.8	5.6	2.7	3.5
Lysozyme	14.3	2.5	50.7	5.6	3.0	3.6
Whey protein	14.3	3.2	50.3	5.6	2.8	3.9
Casein	12.4	2.5	49.4	5.3	2.7	3.5
*glucoside						

On Table 41 only minor differences are found.

Figure 52 Bar chart of Anthocyanins Regent part II

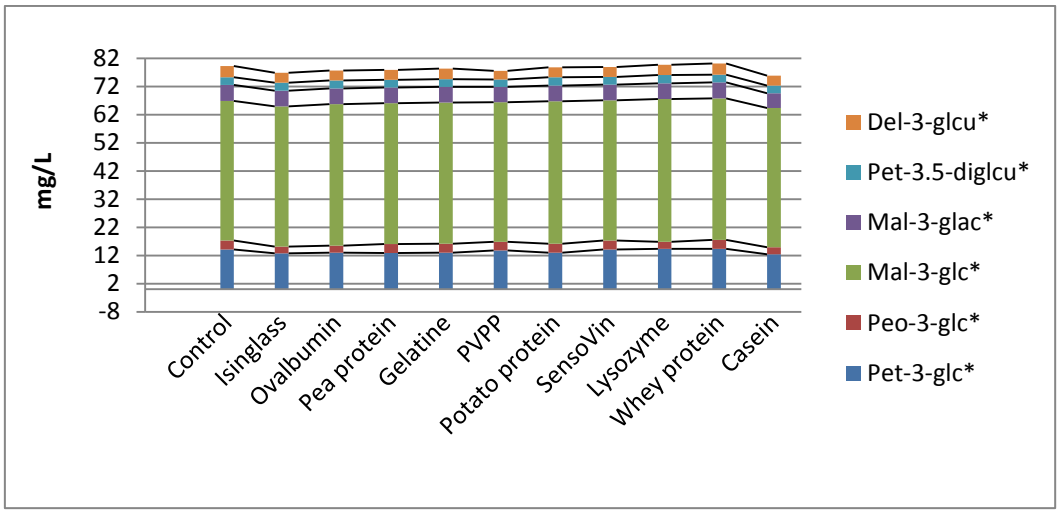


Figure 53 Bar chart of Anthocyanins Regent in % part II

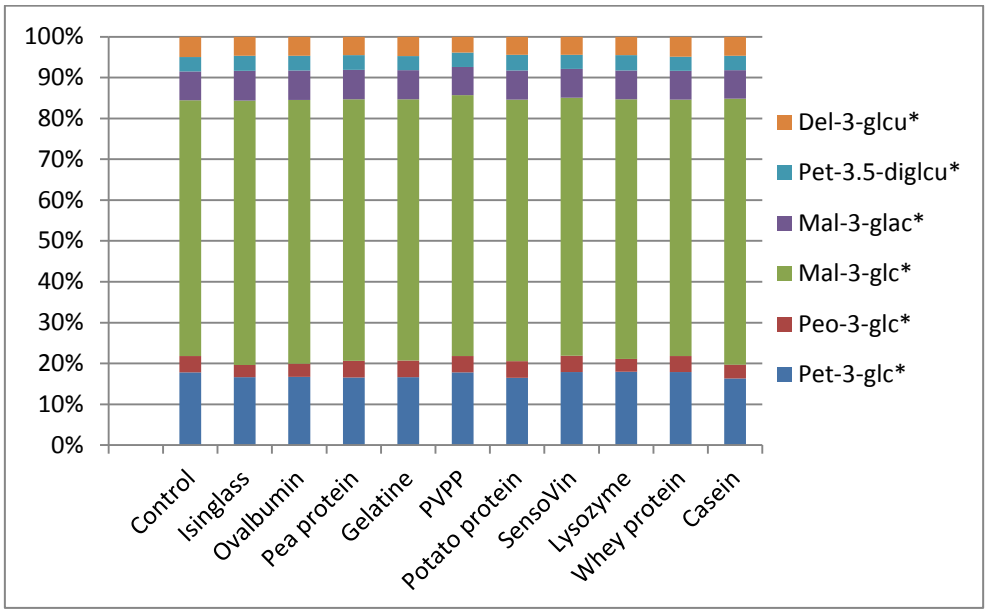


Table 42 Anthocyanins part III Regent - Wine 2

Fining agent/ Sample	**Peo-3.5-diglcu***	**Mal-3.5-diglcu***	**Pet-3-glcu***	**Peo-3-glcu***	**Mal-3-glcu***	**Total Anthocyanins***
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Control	4.8	37.1	2.2	4.3	15.1	**298.1**
Isinglass	4.3	36.4	2.2	4.3	15.0	**293.9**
Ovalbumin	3.0	33.3	2.2	4.2	14.2	**289.9**
Pea protein	4.0	36.0	2.2	4.1	15.4	**298.2**
Gelatine	4.3	36.4	2.2	4.2	14.9	**294.1**
PVPP	4.1	34.3	1.9	3.6	12.9	**289.0**
Potato protein	4.4	35.6	2.2	4.2	12.8	**286.4**
SensoVin	4.3	36.5	2.2	4.2	16.2	**299.4**
Lysozyme	4.2	36.2	2.2	4.2	13.7	**296.9**
Whey protein	4.7	36.7	2.2	4.2	15.3	**298.3**
Casein	4.0	33.9	2.2	4.1	13.8	**284.3**
*glucoside						

On Table 42, casein, ovalbumin, PVPP and potato protein shows a decrease in comparison with control wine.

Figure 54 Bar chart of Anthocyanins of Regent part III

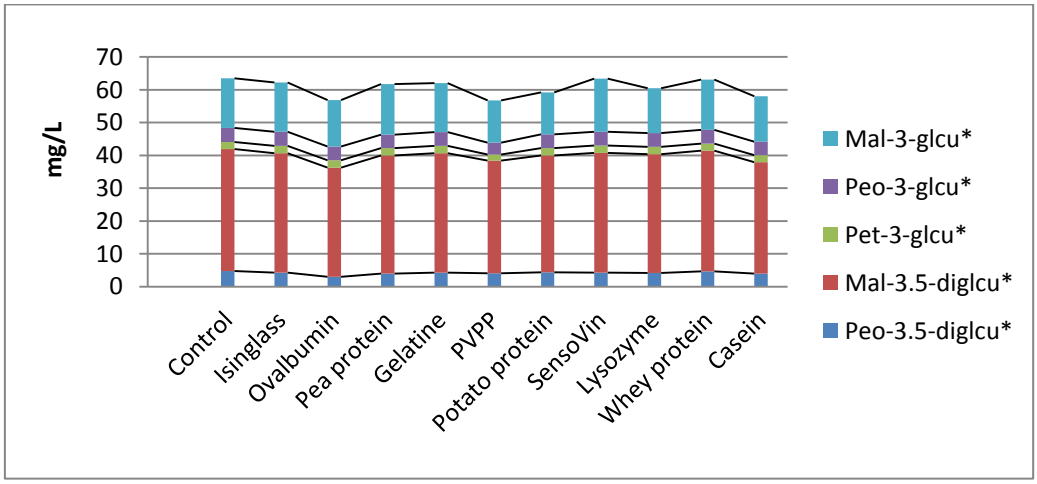
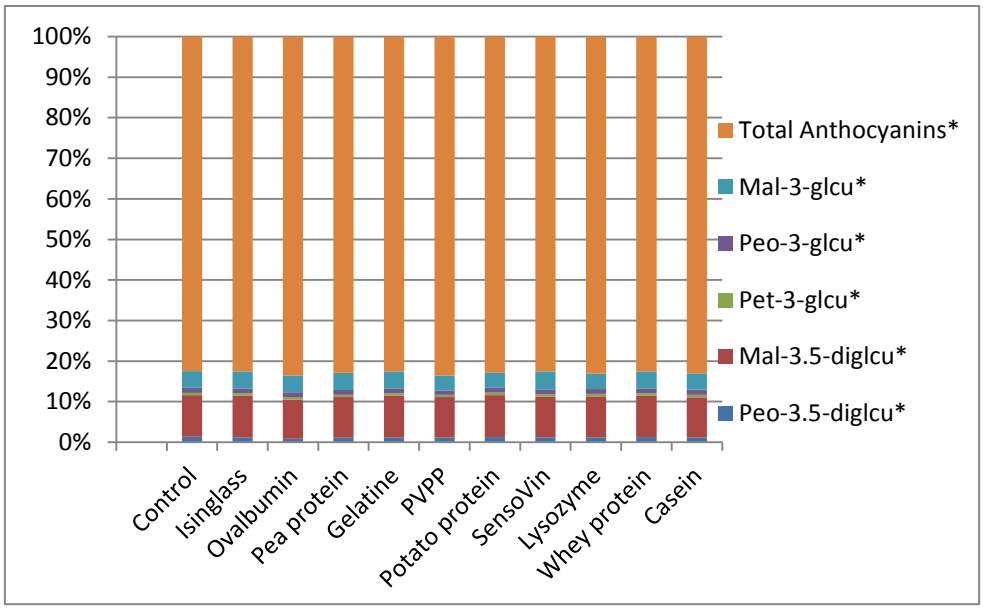
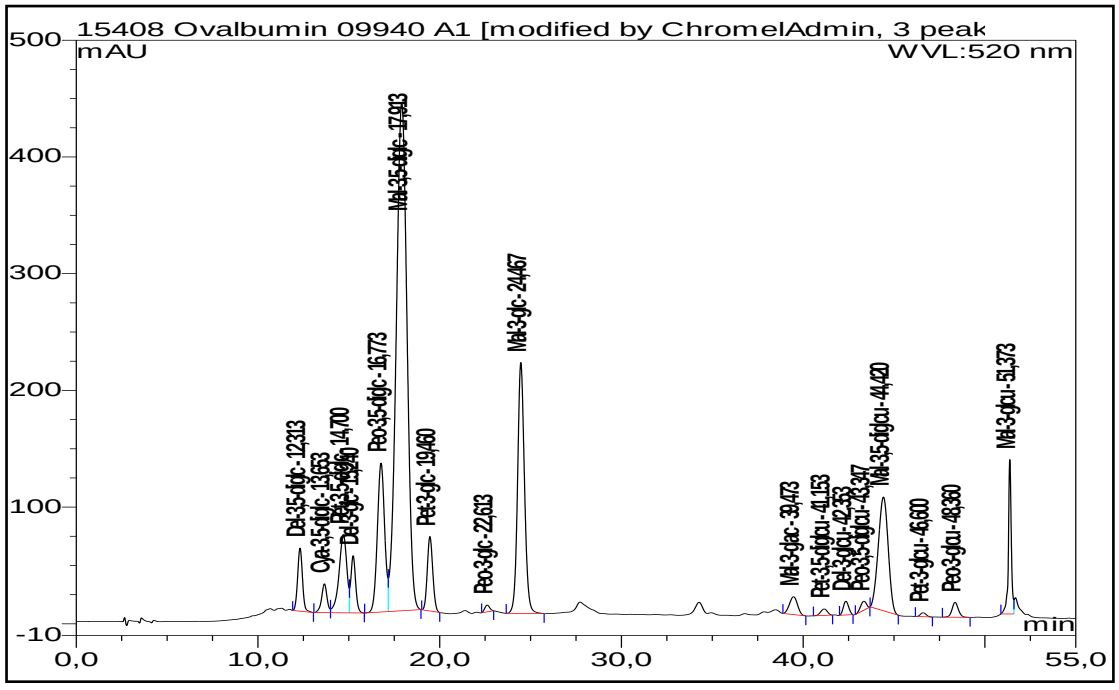


Figure 55 Bar chart of Anthocyanins in % Regent part III

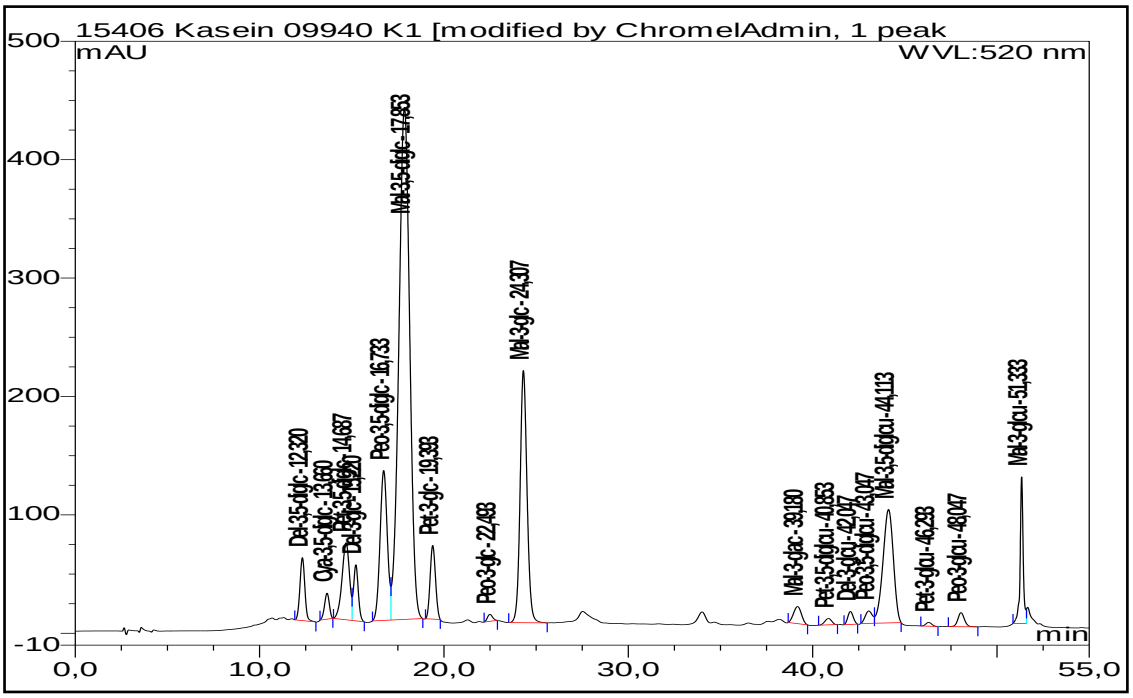


The following three spectrums show the anthocyanins peaks for ovalbumin, casein and lysozyme. All three spectrums have reasonably comparable peaks, meaning that there are no large changes on anthocyanins profile within compared fining agents.

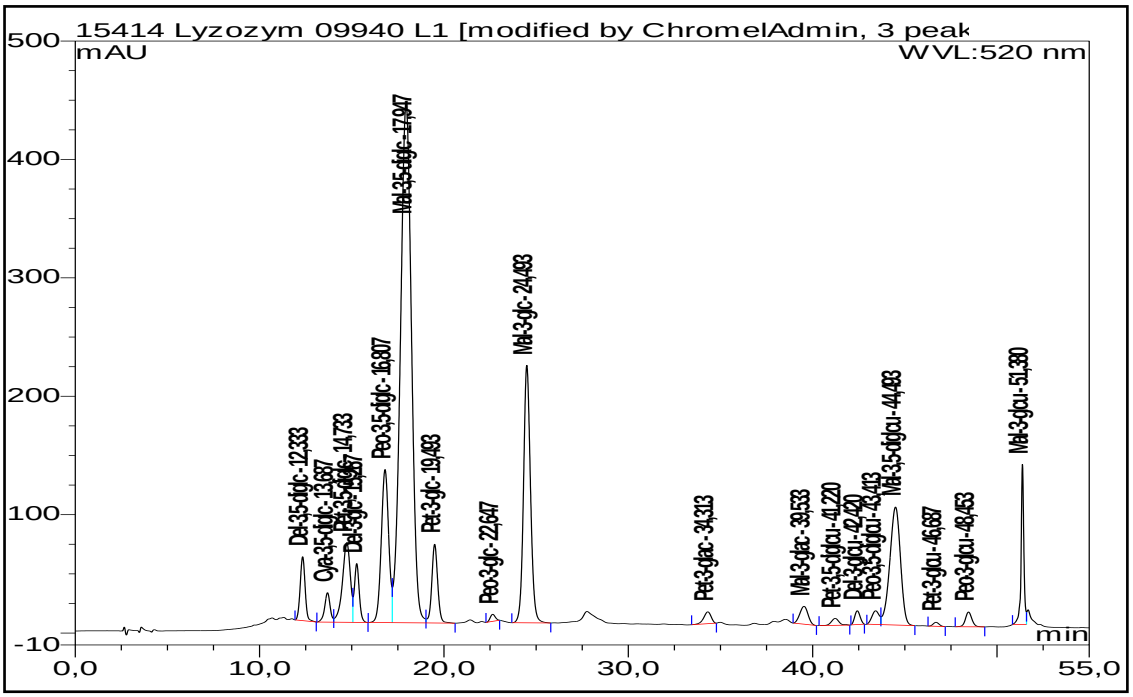
Chromatogram 1 Anthocyanins of wine fined with Ovalbumin Regent -wine 2



Chromatogram 2 Anthocyanins of wine fined with Casein Regent -wine 2



Chromatogram 3 Anthocyanins of wine fined with Lysozyme Regent -wine 2



5.4 Oxidative capacity

Following table contains the TEAC capacity (Trolox equivalent antioxidant capacity) and ORAC (oxygen radical scavenging capacity) of wine 1 and 2 of first trial. The intention was to check the variability of antioxidant capacity with various fining agents used.

On following table there a noticeable difference for wine 2 and PVPP for both TEAC and ORAC. As mentioned above PVPP eliminate tannins, oxidisable cinnamic acids and quinones formed when they oxidise.

Table 43 TEAC and ORAC capacity of wine 1 and 2

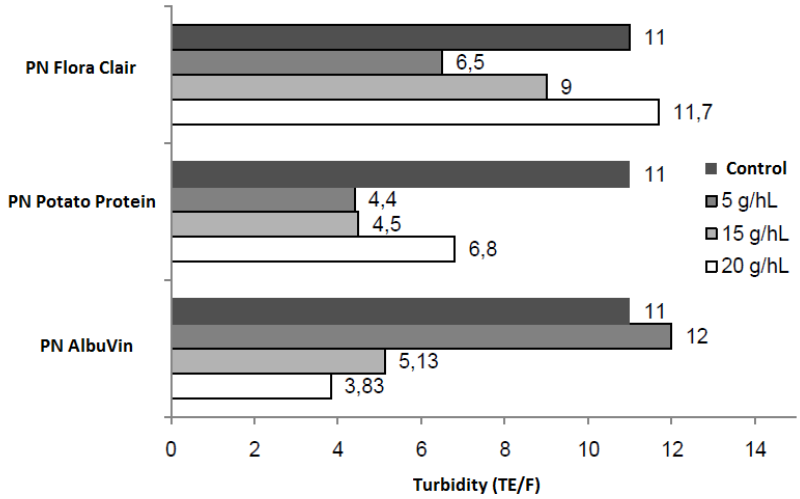
Fining agent/ Sample	Wine 1	Wine 1	Wine 2	Wine 2
	TEAC	**ORAC**	**TEAC**	**ORAC**
	mmoL Trolox/L	**mmoL Trolox/L**	**mmoL Trolox/L**	**mmoL Trolox/L**
Control	4.2	3.6	16.3	24.1
Isinglass	4.0	3.7	14.1	25.1
Ovalbumin	4.0	3.7	14.2	22.5
Pea protein	3.9	3.7	14.3	22.7
Gelatine	4.1	3.6	15.1	23.5
PVPP	3.9	3.1	**12.4**	19.1
Potato protein	4.2	3.6	15.3	22.1
SensoVin	4.1	3.6	14.7	22.5
Lysozyme	4.5	3.6	15.2	24.4
Whey protein	4.4	3.7	15.4	23.2
Casein	3.9	3.4	13.5	25.4

5.5 Turbidity of wines

The turbidity of wine might be crucial on fining trials before the actual fining happens. This trial is one of wine clarity indicator.
Following chart bars are showing the result of turbidity for second trial red wines.
According to Ribéreau-Gayon *et al.* (2006) a red wine has a correspondence between turbidity measurements (NTU) and appearance. A red wine is considered to be turbid when the reading is greater than 8.0 NTU. This trial helped on the selection of fining agent quantity, since one of fining agent aims is the clarity of wine.

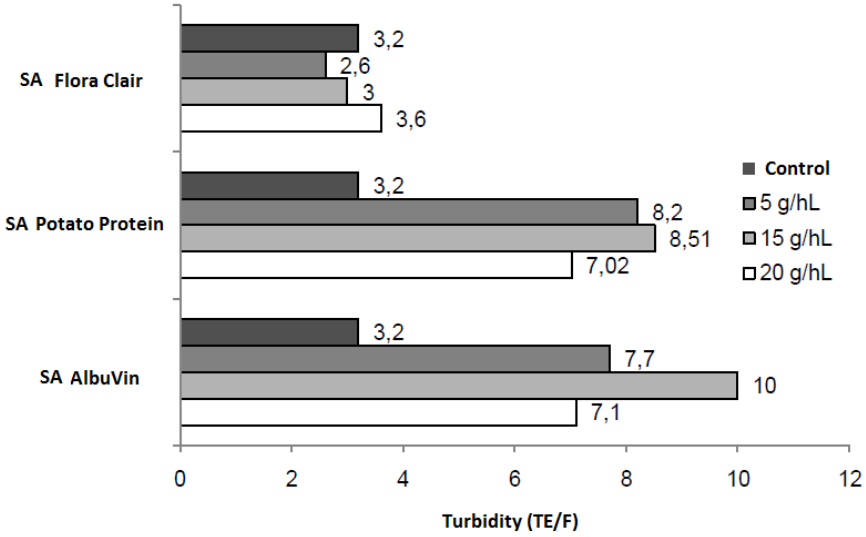
Pinot Noir (PN) wine samples fined with pea protein Flora Clair and potato protein show better clarity when fined with lower dosage. While for ovalbumin is the opposite. The chosen dosage for second trial was therefore of 5g/hL.

Figure 56 Turbidity of Pinot Noir wine



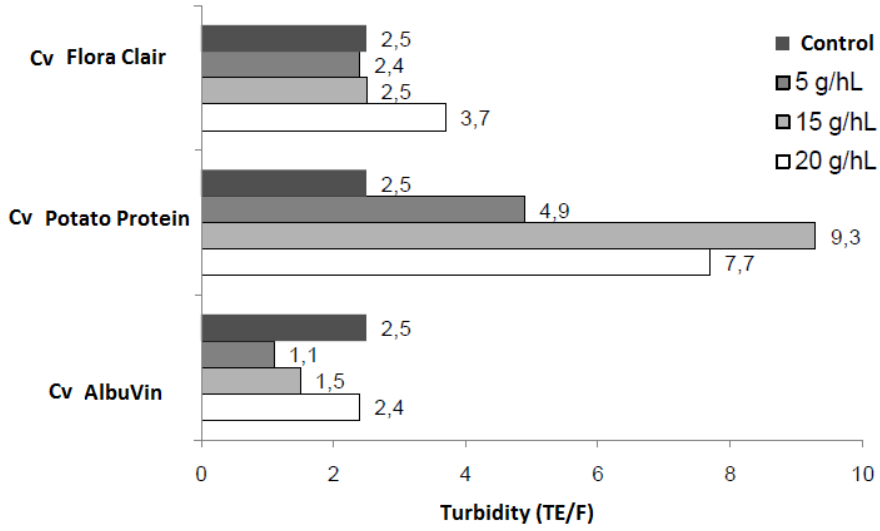
Sangiovese wine samples show better clarity when fined with higher dosage for potato protein as well as for ovalbumin, therefore we use for the main trial 20g/hL.

Figure 57 Turbidity of Sangiovese wine



Cuvée wine samples fined with pea protein Flora Clair and potato protein, as well for ovalbumin show better clarity when fined with lower dosage. The chosen dosage for second trial was therefore of 5g/hL.

Figure 58 Turbidity of Cuvée wine



5.6 Minerals of first trial wine samples

Following tables show the minerals of MT and Regent, wines No. 1 and 2. The process and fining agents are described on first trial of material and methods chapter. Analyses have been done in the laboratory of wine chemistry in the research centre of Geisenheim.

Wines fined with different fining agents have a similar mineral composition, as to be seen on Table 44 and Table 45, only pea protein has a more elevated level of Na if compared with other fining agents and the mineral that varies the most is K.

Table 44 Mineral content of wine 1 – Mueller-Thurgau

Samples	Na	Ca	Mg	Fe	Zn	Cu	K
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Control	5	57	78	0.4	1.0	0.07	879
Isinglass	5	59	78	0.4	1.0	0.08	865
Ovalbumin	8	60	79	0.4	0.9	0.06	884
Pea protein	11	58	78	0.4	1.0	0.05	892
Gelatine	6	58	78	0.4	0.9	0.05	866
PVPP	5	58	78	0.4	1.0	0.08	889
Potato protein	6	58	78	0.4	0.9	0.06	889
SensoVin	8	60	79	0.4	1.0	0.05	874
Lysozyme	6	56	78	0.4	1.0	0.06	878
Whey protein	7	59	78	0.4	0.9	0.05	895
Casein	6	58	78	0.4	1.0	0.05	899

Table 45 Mineral content of wine 2 – Regent

Samples	Na	Ca	Mg	Fe	Zn	Cu	K
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Control	4	48	92	3.2	1.3	0.06	964
Isinglass	6	51	90	3.3	1.4	0.07	967
Ovalbumin	7	51	92	3.6	1.4	0.06	979
Pea protein	10	49	91	3.2	1.4	0.08	980
Gelatine	5	53	91	3.3	1.4	0.06	993
PVPP	6	51	90	3.2	1.4	0.06	979
Potato protein	5	49	90	3.2	1.4	0.06	958
SensoVin	6	50	93	3.2	1.4	0.06	999
Lysozyme	5	48	91	3.2	1.4	< 0.05	983
Whey protein	6	49	92	3.1	1.4	0.06	942
Casein	6	48	92	3.2	1.5	0.11	979

5.6.1 Mineral content of fining material

The mineral content analysis of fining material was done on July of 2011. Standard used for TRFA was selenium 1.0 µg. Mineral content of fining material; pea and potato protein to check its purity.

These results show the difference in mineral content of different producers. Potato proteins are different; they have noteworthy different amounts of Ca, for example. All this small differences may present some changes while fining the wines. The intention here though was only to check the purity of these proteins concerning its minerals, and no problem was found.

Table 46 Mineral content of fining material; pea and potato protein

Sample (part I)	Sample amount	K	Ca	Ti	Mn	Fe	Cu
		µg	µg	µg	µg	µg	µg
Potato protein_1	10 µg	156 µg	841 µg	28 µg	2 µg	160 µg	6 µg
Potato protein_2	10 µg	147 µg	856 µg	26 µg	2 µg	156 µg	6 µg
Pea protein_1	9 µg	1313 µg	289 µg		8 µg	171 µg	4 µg
Pea protein_2	9 µg	1301 µg	291 µg		8 µg	164 µg	4 µg
Potato protein_Begerow_1	12 µg	93 µg	539 µg	29 µg		147 µg	5 µg
Potato protein_Begerow_2	12 µg	83 µg	525 µg	30 µg		148 µg	6 µg
Sample (part II)	**Sample amount**	**Cu**	**Zn**	**Br**	**Sr**	**W**	**Pb**
		µg	µg	µg	µg	µg	µg
Potato protein_1	10 µg	6 µg	1 µg	4 µg	46 µg	4 µg	1 µg
Potato protein_2	10 µg	6 µg	2 µg	4 µg	49 µg	4 µg	1 µg
Pea protein_1	9 µg	4 µg	72 µg	1 µg			
Pea protein_2	9 µg	4 µg	71 µg	2 µg	2 µg		
Potato protein_Begerow_1	12 µg	5 µg	1 µg	3 µg	34 µg	6 µg	1 µg
Potato protein_Begerow_2	12 µg	6 µg	1 µg	4 µg	34 µg	5 µg	1 µg

5.7 Colour measurement CIELab

Colour measurement was used here as a parameter to evaluate effect of fining on wine between different (animal and vegetal) fining agents.

When a wine is fined some changes will normally occur on the phenol content, wine chromatic characteristics are also related to its phenolics.

Each wine sample was poured into individual cuvettes and analysed with the spectrophotometer.

Figure 59 CIELab bars chart analysis of fined wines

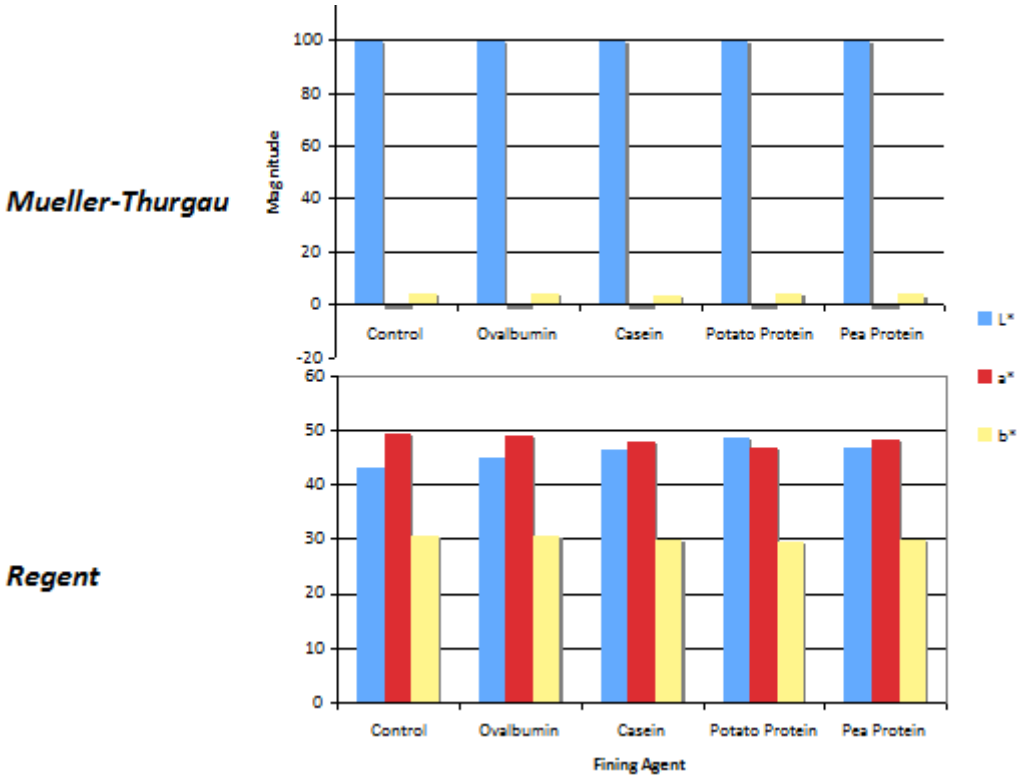
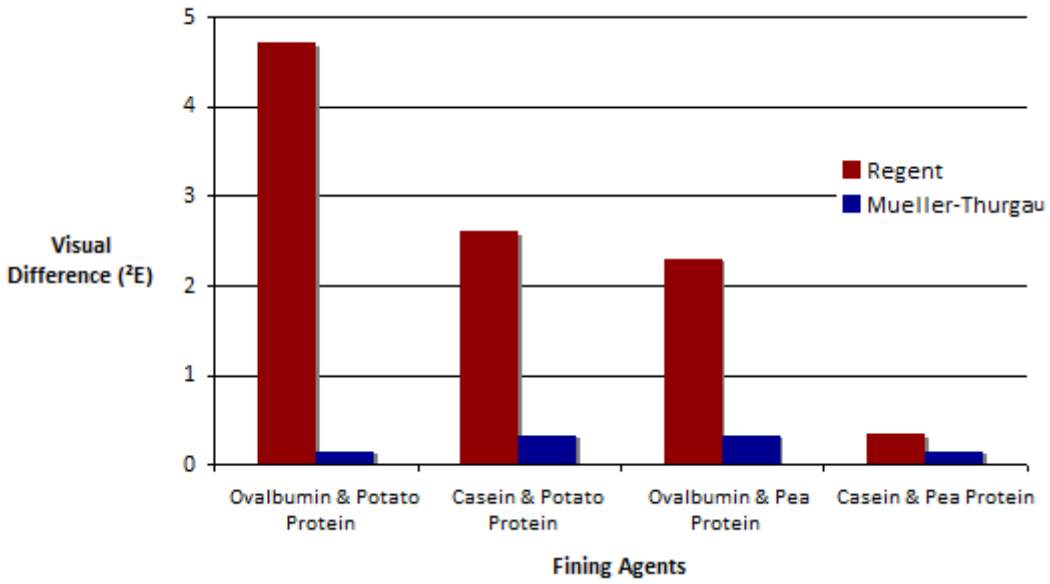


Figure 59 charts for MT wine there is no significant difference on results. This could be explained by low phenolic content of the wine. However the intensity of colour (A_{420}) and the b*coordinate (indicating the position on axe yellow) have slightly decreased, especially for casein, which revealed a decrease in yellow colour. Casein is widely used on white wines, because it reduces yellowness, normally caused by phenol oxidations (Troost, 1988).

Similar results and no significance have been found in other studies done using vegetal proteins (Mira *et al.*, 2006).

Regent wine shows statistical significance as to be seen on following figure.

Figure 60 CIELab derived visual difference of Regent and Mueller-Thurgau wines



When compared to one another ovalbumin and both plant proteins, casein and potato protein had significant deviations in colour that could be detected visually by humans (when $\Delta E > 1$ as by formula mentioned on previous material and methods chapter). No differences in colour between casein and pea protein can be detected visually as to be seen on Figure 60.

These results are similar to what Mira *et al.* (2006) found on her study results and behaviour on red wines treated with plant proteins according to chromatic intensity, tonality and lightness. On that study the more vegetal proteins were used the lower the tonality a* and b* CIELab coordinates. These are positive results to protein efficacy on fining.

5.8 Sensory analysis

5.8.1 First trial wines

Here is important to bear in mind that the wines have been strongly over-fined, as a result wines could not properly fulfil the necessary duties for an appropriate tasting.

First tasting

The intention of this first tasting was to compare fining agents that may be declared on the label with other that does not, therefore alternatives, aiming to check if they are comparable on a tasting level.

Ranking test for Mueller-Thurgau, wine No.1. There are no significant differences between tasted wines. There are though some tendencies; control wine is considered to be the less fruity on wine flavour, while potato protein fined wine is the fruitiest.
Cosme *et al.* (2011) recently researched the possibility of using non-allergenic pea protein or PVPP as alternatives for wine fining. Globally the study comes to good and significant results; but on sensorial analysis no difference ($p>0.05$) was found among wine samples set with different fining agents, just as in this work.

Figure 61 Average attribute intensity for each fining agent (MT)

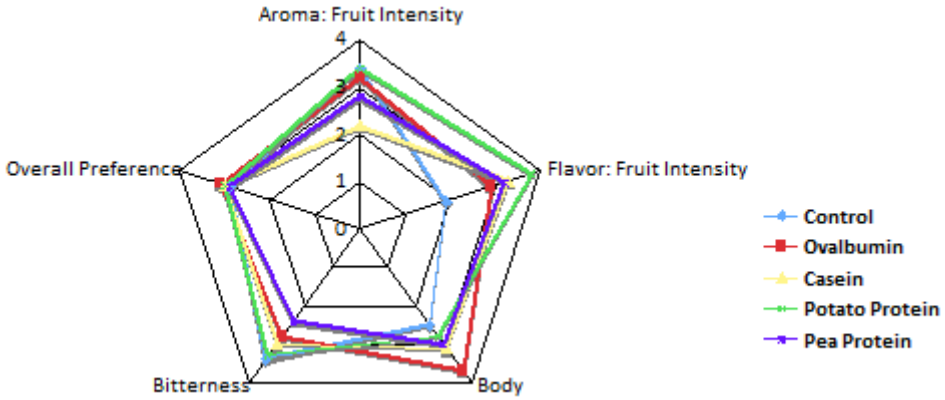


Table 47 Average rank of Mueller-Thurgau wines for each attribute's intensity

	Control	Ovalbumin	Casein	Potato protein	Pea Protein
Aroma: Fruit intensity	3.4 [a]	3.2 [a]	2.2 [a]	3.4 [a]	2.8 [a]
Flavour: Fruit intensity	1.9 [a]	2.9 [a]	3.3 [a]	3.8 [a]	3.2 [a]
Body	2.5 [a]	3.7 [a]	3.1 [a]	2.8 [a]	3.0 [a]
Bitterness	3.4 [a]	2.8 [a]	3.0 [a]	3.3 [a]	2.4 [a]
Overall preference	2.9 [a]	3.1 [a]	3.0 [a]	3.0 [a]	2.9 [a]

*Any two averages not followed by the same letter are significantly different (α=0.05)

Ranking test for Regent, wine No. 2. Here, for a second time, there are no significant differences, but some preferences. The overall preference is for pea protein fined wine.

Figure 62 Average attribute intensity for each fining agent in Regent

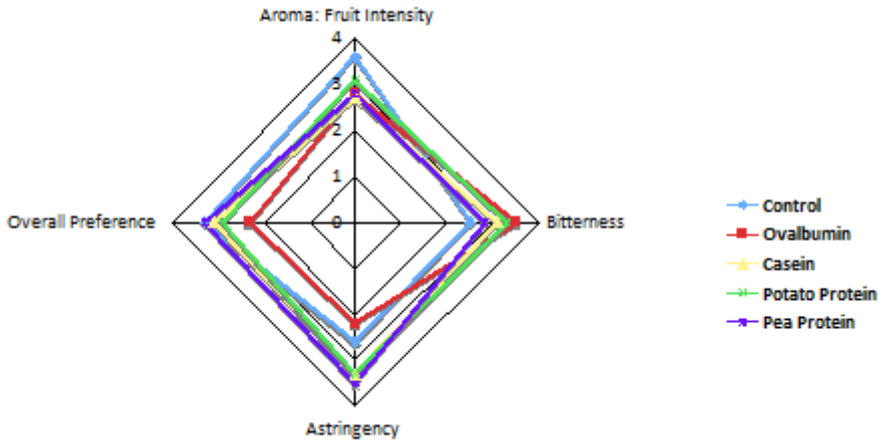


Table 48 Average rank of Regent for each attribute's intensity

	Control	Ovalbumin	Casein	Potato protein	Pea Protein
Aroma: Fruit intensity	3.6 [a]	2.8 [a]	2.7 [a]	3.1 [a]	2.8 [a]
Bitterness	2.5 [a]	3.5 [a]	3.1 [a]	3.3 [a]	2.8 [a]
Astringency	2.6 [a]	2.2 [a]	3.3 [a]	3.3 [a]	3.5 [a]
Overall preference	3.3 [a]	2.3 [a]	3.1 [a]	2.9 [a]	3.3 [a]

*Any two averages not followed by the same letter are significantly different (α=0.05)

Second tasting

On second tasting the intention was to check the differences on a sensory level, between different fining agents and separation methods. Therefore results are found in groups of similar fining agent compared with all separation methods together.

Mueller-Thurgau wine groups

Results are only considered significant when α=0.05.

Table 49 Results of first group of white samples (14th April 2011)

Sum of ranks (Friedman) Number of tasters: 19								
	1st Ranking (1-5)					**2nd Ranking (1-3)**		
	Sterile filtration	0.45 µm Membrane	Cross-Flow	Centrifuge	Earth filter	Silica sol	Pasteuri-sation	Bento-nite
Control wine	47	61	62	55	60	34	40	40
Lysozyme	51	52	64	57	61	38	36	40

Table 49 shows that in the first ranking, control sample, the preferred wine by tasters was the one that passed through sterile filtration. Cross-flow was the least appreciated the same happened for lysozyme, but none of the results are statistically relevant.
For the second ranking, control sample, silica sol was better rated and the less rated was pasteurisation. For lysozyme samples the better rated is pasteurisation is the less rated is bentonite, but again the sum of these results are not statistically significant, as to be seen on Table 50.

Table 50 Friedman's test first group, white wine

Friedman test	R1 Control	R1 Lysozyme	R2 Control	R2 Lysozyme
Sum of squares of sums of ranks	163	163	4356	434
F	3.24	2.65	1.26	0.421
corrected F	3.24	2.65	1.26	0.421
Significance F (Risk)	0.51	0.61	0.53	0.810
Significance corrected F (Risk)	0.51	0.61	0.53	0.810

Table 51 Results of second group of white samples (15th April 2011)

Sum of ranks (Friedman) number of tasters: 16								
	1st Ranking (1-5)					**2nd Ranking (1-3)**		
	Sterile filtration	0.45 µm Membrane	Cross-Flow	Centri-fuge	Earth filter	Silica sol	Pasteuri-sation	Bentonite
Ovalbumin	47	36	38	34	40	**21**	24	33
Isinglass	37	38	45	36	39	27	23	28
Gelatine	44	41	44	25	41	27	28	23

Table 51 First ranking shows that for ovalbumin, isinglass and for gelatine samples the best rated method is the centrifuge, but with no statistical relevance for any of these samples. Whereas on the second ranking there is relevance for ovalbumin, the preferred method is silica gel fining with 95% of significance level (Table 52). No other significant results for any of the other agents on the second group.

Table 52 Friedman's test second group, white wine

Friedman test	R1 Ovalbumin	R1 Isinglass	R1 Gelatine	R2 Ovalbumin	R2 Isinglass	R2 Gelatine
Sum of squares of sums of ranks	7705	7655	7859	2106	2042	2042
F	3.076	1.538	7.815	6	1.07	1.07
corrected F	3.076	1.538	7.815	6	1.07	1.07
Significance F (Risk)	0.54	0.819	0.098	**0.0498**	0.58	0.58
Significance corrected F (Risk)	0.54	0.819	0.098	**0.0498**	0.58	0.58

Table 53 Results of third group of white samples (12th May 2011)

Sum of ranks (Friedman) number of tasters:12								
	1st Ranking (1-5)					**2nd Ranking (1-3)**		
	Sterile filtration	0.45 µm Membrane	Cross-Flow	Centri-fuge	Earth filter	Silica sol	Pasteuri-sation	Bentonite
Whey protein	32	30	41	37	39	24	27	21
Casein	**24**	47	41	33	34	24	26	21
SensoVin	40	28	41	29	42	21	22	29

Table 53 shows all milk proteins used in this study. For the first ranking whey protein sample is the best method according to the tasters and the least appreciated was Cross-flow, the same happened for SensoVin for the most appreciated while the least was Earth filtration, but neither whey protein nor SensoVin results are statistically significant. For Casein the best sample is sterile filtration and the least rated is membrane with a significance of 94% to be seen in orange colour (Table 54). On the second ranking no statistically relevance has been found.

Table 54 Friedman's test third group, white wine

Friedman test	R1 Whey protein	R1 Casein	R1 SensoVin	R2 Whey protein	R2 Casein	R2 SensoVin
Sum of squares of sums of ranks	6575.5	6782.5	6670.0	1746.0	1743.5	176
F	3.1833	10.08	6.3333	1.500	1.2917	3.166
corrected F	3.1967	10.12	6.3333	1.500	1.3191	3.166
Significance F (Risk)	0.5276	**0.039**	0.1756	0.472	0.5242	0.205
Significance corrected F (Risk)	0.5255	**0.038**	0.1756	0.4724	0.5171	0.205

Table 55 Results of fourth group of white samples (13th May 2011)

Sum of ranks (Friedman) number of tasters:13								
	1st Ranking (1-5)					**2nd Ranking (1-3)**		
	Sterile filtration	0.45 µm Membrane	Cross-Flow	Centrifuge	Earth filter	Silica sol	Pasteuri-sation	Bentonite
Potato protein	**25**	31	48	46	45	21	29	28
Pea protein	38	35	39	37	46	24	25	29
PVPP	37	39	44	36	39	29	24	25

Table 56 Friedman's test fourth group, white wine

Friedman test	R1 Potato protein	R1 Pea protein	R1 PVPP	R2 Potato protein	R2 Pea protein	R2 PVPP
Sum of squares of sums of ranks	8031.0	7675.0	7643.0	2066.0	2042.0	2042.0
F	13.10	2.15	1.16	2.923	1.07	1.07
corrected F	13.10	2.15	1.16	2.923	1.07	1.07
Significance F (Risk)	**0.01**	0.70	0.88	0.231	0.58	0.58
Significance corrected F (Risk)	**0.01**	0.70	0.88	0.231	0.58	0.58

Table 55 shows plant proteins used on this study and PVPP. For the first ranking of potato protein, the best method according to the tasters is sterile filtration and the least appreciated was cross-flow, with statistical significance of 99%. On the second ranking there is no statically significance to the favourite silica sol.

For pea protein the most appreciated was 0.45 µm membrane and the last was again cross-flow for the first ranking. On the second the most appreciated was silica sol and bentonite was the last with no significance in statistics.
For PVPP first ranking centrifuge is the best rated sample and cross-flow the worst, on second ranking pasteurization is the favourite and silica sol the worst rated, but no statistical significance between the samples.

Final remark for white wines there is no clear relation between different methods of filtration within each fining material, although fine filtration is often the best rated there is no statistically significance to support this tendency.

Regent wine groups

Results are only considered significant when α=0.05. Control is present in all tests as to be seen on Table 58 or on following table of every rank test.

Table 57 Results of fourth group of red samples (13th May 2011)

Sum of ranks (Friedman) Number of tasters: 17

	1st Ranking (1-5)					2nd Ranking (1-3)		
	Sterile filtration	0.45µm Membra-ne	Cross-Flow	Centrifuge	Earth filter	Silica sol	Pasteuri-sation	Bentonite
Control wine	48	42	50	37	48	31	28	31
Lyso-zyme	46	52	40	42	45	27	33	30

On Table 57 for first and second ranking there is no statistical significance (Table 58), although centrifugation was the preferred method for control wine and cross-flow for lysozyme, both on first ranking. For the second ranking control, pasteurisation was the one more panellists liked, whereas for lysozyme it was silica sol.

Table 58 Friedman's test first group, red wine

Friedman test	R1 Control	R1 Lysozyme	R2 Control	R2 Lysozyme
Sum of squares of sums of ranks	10241.0	2706.0	10209.0	2718.0
F	3.0933	0.4000	2.2400	1.2000
corrected F	3.0933	0.4000	2.2400	1.2000
Significance F (Risk)	0.5423	0.8187	0.6917	0.5488
Significance corrected F (Risk)	0.5423	0.8187	0.6917	0.5488

Table 59 Results of second group of red samples (27th May 2011)

Sum of ranks (Friedman) number of tasters: 16

	1st Ranking (1-5)					**2nd Ranking (1-3)**		
	Sterile filtration	0.45 µm Membrane	Cross-Flow	Centri-fuge	Earth filter	Silica sol	Pasteuri-sation	Bentonite
Ovalbumin	38	48	52	53	49	30	32	34
Isinglass	45	49	50	53	43	30	31	35
Gelatine	51	58	36	45	50	34	31	31

Although Table 59 shows that sterile filtration was the favourite for ovalbumin, earth filter for isinglass and cross-flow for gelatine, none of these results are significantly for the statistics, neither for the second ranking.

Table 60 Friedman's test second group, red wine

Friedman test	R1 Ovalbumin	R1 Isinglass	R1 Gelatine	R2 Ovalbumin	R2 Isinglass	R2 Gelatine
Sum of squares of sums of ranks	11662.0	11584.0	11786.0	3080.0	3086.0	3078.0
F	3.5500	1.6000	6.6500	0.5000	0.8750	0.3750
corrected F	3.5500	1.6000	6.6500	0.5000	0.8750	0.3750
Significance F (Risk)	0.4703	0.8088	0.1556	0.7788	0.6456	0.8290
Significance corrected F (Risk)	0.4703	0.8088	0.1556	0.7788	0.6456	0.8290

Table 61 Results of third group of red samples (9th June 2011)

Sum of ranks (Friedman) number of tasters: 21

	1st Ranking (1-5)					2nd Ranking (1-3)		
	Sterile filtration	0.45 µm Membrane	Cross-Flow	Centrifuge	Earth filter	Silica sol	Pasteurisation	Bentonite
Whey protein	54	52	44	41	49	33	30	33
Casein	57	37	51	**35**	60	35	27	34
SensoVin	49	42	51	45	53	36	30	30

On the first ranking of Table 61 only one fining agent, which is casein has a significant difference of 99% where panellists preferred centrifuge (Table 62). No other significance on neither from ranking tests.

Table 62 Friedman's test third group, red wine

Friedman test	R1 Whey protein	R1 Casein	R1 SensoVin	R2 Whey protein	R2 Casein	R2 SensoVin
Sum of squares of sums of ranks	11638.0	12044.0	11600.0	3078.0	3110.0	3096.0
F	2.9500	13.1000	2.0000	0.3750	2.3750	1.5000
corrected F	2.9500	13.1000	2.0000	0.3750	2.3750	1.5000
Significance F (Risk)	0.5662	**0.0108**	0.7358	0.8290	0.3050	0.4724
Significance corrected F (Risk)	0.5662	**0.0108**	0.7358	0.8290	0.3050	0.4724

Table 63 Results of fourth group of red samples (7th July 2011)

Sum of ranks (Friedman) number of tasters:

	1st Ranking (1-5)					2nd Ranking (1-3)		
	Sterile filtration	0.45 µm Membrane	Cross-Flow	Centrifuge	Earth filter	Silica sol	Pasteurisation	Bentonite
Potato protein	39	37	48	31	40	27	28	23
Pea protein	30.5	43.5	31	45	45	19	29	30
PVPP	43	33	46.5	39	34.5	25	25	28

On the last appointment tasters preferred the centrifuge for potato protein, sterile filtration for pea protein and membrane for PVPP. No significance though was found on the first ranking neither on the second.

Table 64 Friedman's test fourth group, red wine

Friedman test	R1 Potato protein	R1 Pea protein	R1 PVPP	R2 Potato protein	R2 Pea protein	R2 PVPP
Sum of squares of sums of ranks	7755.0	7833.5	7734.5	2042.0	2102.0	2034.0
F	4.6154	7.0308	3.9846	1.0769	5.6923	0.4615
corrected F	4.6154	7.0579	4.0155	1.0769	5.6923	0.4615
Significance F (Risk)	0.3291	0.1343	0.4081	0.5836	0.0581	0.7939
Significance corrected F (Risk)	0.3291	0.1329	0.4039	0.5836	0.0581	0.7939

Here, a final overview of all tables above, which are comparing the influence of filtering method within every fining agent.
As one may conclude from Table 65, there are not many significant results and the few that are present cannot be related to each other.

1st Ranking-test: sterile filtration, membrane filter, diatomaceous filter, cross-Flow and centrifuge
2nd Ranking-test: flash-pasteurisation, bentonite and silica sol.

Table 65 Overview of first year tasting results

	Fining material	Concentration	1st Ranking-test*	2nd Ranking-test*	Tasting day
White wine	Control wine	0	ns	ns	14th April
	Lysozyme	50 g/hL	ns	ns	14th April
	Ovalbumin	16 g/hL	ns	**Silica-sol**	15th April
	Isinglass	6 g/Ll	ns	ns	15th April
	Gelatine	10 g/hL	ns	ns	15th April
	Whey protein	44 g/hL	ns	ns	12th May
	Casein	40 g/hL	**Sterile filtration**	ns	12th May
	SensoVin	50 g/hL	ns	ns	12th May
	Potato protein	30 g/hL	**Sterile filtration**	ns	13th May
	Pea protein	30 g/hL	ns	ns	13th May
	PVPP	60 g/hL	ns	ns	13th May
Red wine	Control wine	0	ns	ns	26th May
	Lysozyme	50 g/hL	ns	ns	26th May
	Ovalbumin	16 g/hL	ns	ns	27th May
	Isinglass	6 g/Ll	ns	ns	27th May
	Gelatine	10 g/hL	ns	ns	27th May
	Whey protein	44 g/hL	ns	ns	9th June
	Casein	40 g/hL	**Centrifuge**	ns	9th June
	SensoVin	50 g/hL	ns	ns	9th June
	Potato protein	30 g/hL	ns	ns	7th July
	Pea protein	30 g/hL	ns	ns	7th July
	PVPP	60 g/hL	ns	ns	7th July
	* Only with significance 0.05. Ns: not significant				

5.8.2 Second trial wines

Wine numbers: 3 to 8, to be found on Table 76 and Table 77. This is an improved repetition of first year tasting, where wines are now not over-fined and could be better analyzed on a sensory view.

Triangle test

By using the triangle test every fined wine has been tested against its control wine, i.e. not fined, aiming to check if tasters could find any difference between them. On Table 50 only wines with significance of ≥5% are taken into account and are bold marked. In

this tasting at least 12 out of 23 total tasters have to have the same answer to fit on the 0.05 significance.

Only 2 wines; a white cuvée and the Sangiovese have significant results, both for the same fining agent, FloraClair or namely pea protein.

Table 66 Triangle sensory test of second year wines (No.3 to 8)

Wine	Fining agent	Significance
First triangle test		
White Cuvée Wine no.4	Casein	0.2413
	FloraClair (Pea Protein)	**0.0347***
	Potato protein	0.10
Second triangle test		
Mueller-Thurgau Wine no.5	Casein	0.45
	FloraClair (Pea Protein)	0.24
	Potato protein	0.68
Third triangle test		
Riesling Wine no.3	Casein	0.45
	FloraClair (Pea Protein)	0.68
	Potato protein	0.68
Fourth triangle test		
Red Cuvée Wine no.6	AlbuVin (Albumin)	0.35
	FloraClair (Pea Protein)	0.69
	Potato protein	0.97
Fifth triangle test		
Pinot Noir Wine no.7	AlbuVin (Albumin)	0.52
	FloraClair (Pea Protein)	0.21
	Potato protein	0.83
Sixth triangle test		
Sangiovese Wine no.8	AlbuVin (Albumin)	0.11
	FloraClair (Pea Protein)	**0.049***
	Potato protein	0.92
*significantly different (α=0.05) Number of tasters: whites 13 and reds 23		

A simple ranking test has been done together with the triangle test, Table 67, no significant difference has been found.

Table 67 Average rank of red wines for each fining agent and control

	Control	Ovalbumin	Potato protein	Pea Protein
Red Cuvée	3.7[a]	2.3[a]	3.1[a]	3.0[a]
Pinot Noir	2.8[a]	2.8[a]	3.5[a]	2.9[a]
Sangiovese	3.5[a]	2.9[a]	2.7[a]	2.9[a]

*Any two averages not followed by the same letter are significantly different (α=0.05)

5.9 Wine conductivity related to lysozyme and metatartaric acid

This trial was conducted on the Research Centre of Geisenheim, the intention here was to check if the presence of lysozyme and metatartaric acid together change the conductivity of wine, which is directly related to wine stability that can be measured by µS or by a conductivity-meter, as previous explained on material and methods.
Weiand (2004) has reported that metatartaric acid precipitates lysozyme Metatartaric acid is known to raise wine stability. As questions have been posed regarding the residues of lysozyme when they are used together, this trial is an attempt to check if lysozyme would inhibit metatartaric or vice-versa, by measuring the wine conductivity, or indirectly, its stability.

Wines treated with lysozyme and metatartaric acid
Following tables show the conductivity of wines No. 9 and 10 of second trial, as shown in material and methods chapter.

- White: Riesling and red: Sangiovese

The quantity of lysozyme added in red and white wine was 50 g/hL (500 ppm). Metatartaric acid was added in the amount of 10 g/hL and the quantity of bentonite was:

- 200 g/hL for red wine
- 350 g/hL for white wine

Table 68 Stability of wine by mini-contact method

	Red wine	White wine
Very stable	30 µS	25 µS
Stable	50 - 60 µS	24 - 40 µS
Unstable	60 - 70 µS	40 - 60 µS
Absolutely unstable	>70 µS	>60 µS

(Source: modified from Müller, 1976)

Following table shows that Sangiovese control wine is unstable and gets even less stable when lysozyme is added. When bentonite is added the stability comes slightly back and lastly it goes to very stable when metatartaric acid is added.
In red wines lysozyme reacts with phenols, as it has been discussed above in this work and presented on Figure 73 (Martin Vialatte, 2013). As an explanation for the raise in instability of wine after lysozyme addition is that the phenols that act as colloid protectors (Brunner *et al.*, 2013) are removed leaving the wine less stable. Further addition of metatartaric acid brings back wine tartaric stability.

While for white wine Riesling lysozyme turns the control wine which was stable in very stable. The addition of bentonite does not change much the numbers and finally wines with metatartaric acid are more stable than the control wine, but less than wines only with lysozyme and lysozyme followed by bentonite fining. These results can be found as bar charts on Figure 63and Figure 64.
Lysozyme acts in all probability in colloidal solution as a protector, since it is highly soluble in wine pH due to its Ip and low MW. Further addition of metatartaric acid a reduction in lysozyme content happens, as demonstrated in this work on ELISA results and by Figure 74, to Figure 78.

Table 69 Conductivity of wines 9 and 10 of trial 2

Wine	Sample	Measure 1	Measure 2	Measure 3	Mean	Deviation
Riesling	Control	34 µS	29 µS	29 µS	31 µS	2.89
	Lysozyme	13 µS	13 µS	13 µS	13 µS	0
	Lysozyme + Bentonite	15 µS	15 µS	12 µS	14 µS	1.73
	Lys. + Ben. + Metatartaric acid	27 µS	27 µS	27 µS	27 µS	0
Sangiovese	Control	89 µS	84 µS	85 µS	86 µS	2.65
	Lysozyme	119 µS	125 µS	117 µS	120 µS	4.16
	Lysozyme + Bentonite	109 µS	104 µS	117 µS	110 µS	6.56
	Lys. + Ben. + Metatartaric acid	27 µS	27 µS	27 µS	27 µS	0

On following figures the higher the conductivity number is the lower is the stability.

Figure 63 Bars chart of conductivity means (µS) in wine No. 9 – Riesling (N:3)

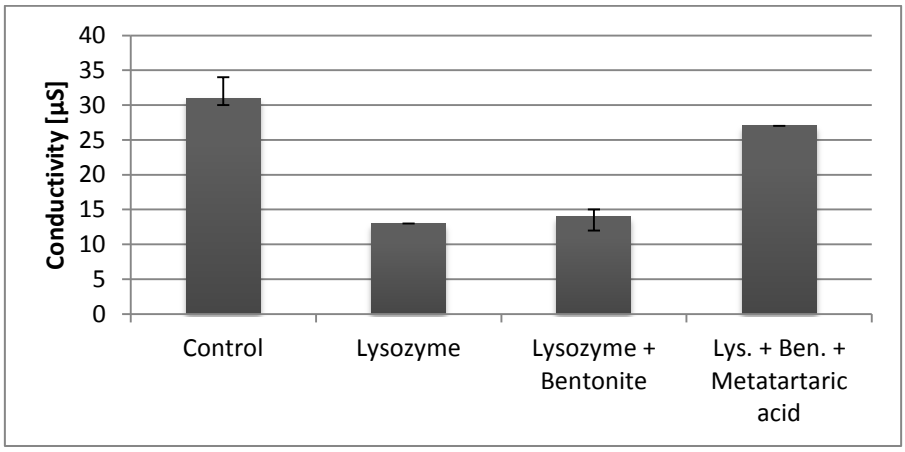
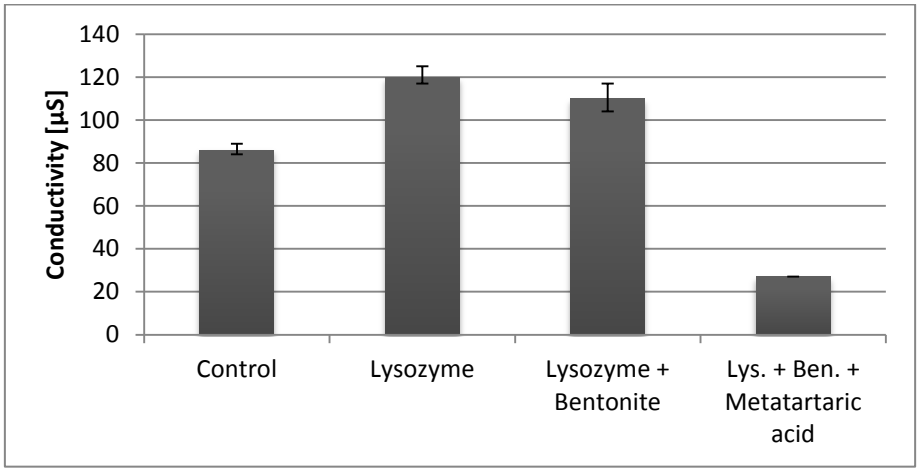


Figure 64 Bar chart of conductivity means (µS) in wine No. 10 –Sangiovese (N:3)



Wines were stored under different temperatures; in warming cabinet and in cold storage warehouse, as explained in material and methods, due to metatartaric acid thermo labile properties, Table 12. The conductivity was measured again after two storing weeks, as it can be seen in following Table 70. This temperature changes were applied to mimic wine transport or temperature changes in warehouses since metatartaric acid is well known to be temperature labile.

On following table it is clear to see that red Sangiovese wines kept on constant colder temperatures (sample RKKL) have higher stability, because metatartaric acid is still present and acting as a protector. Wines that passes through constant temperature

changes between 17°C and 30°C (sample Rabw) are even less stable than wine that had higher but constant temperature of 30°C (sample R30).
White Riesling, kept on same temperature is slightly less stable than wine that changed constantly temperature and wines kept under higher temperatures are the less stable of them. This wine goes back to the same stability of control wine 30µS = stable.

Table 70 Conductivity of wines 9 &10, trial 2 with different storage temperatures

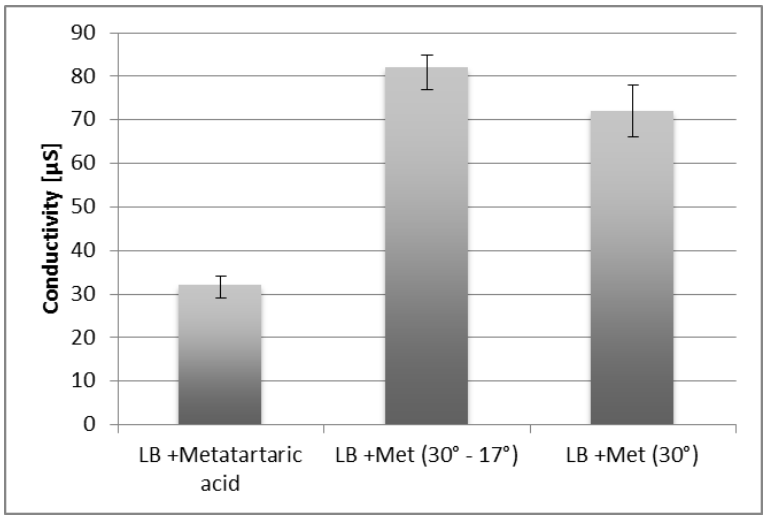
Wine	Codes*	Variant	Measure 1	Measure 2	Measure 3	Mean	Deviation
Sangiovese	RKKL	LB +Metatartaric acid (17°C)	32 µS	29 µS	34 µS	**32 µS**	2,52
	Rabw	LB +Met (30° - 17°)	85 µS	77 µS	84 µS	**82 µS**	4,36
	R30	LB +Met (30°)	66 µS	73 µS	78 µS	**72 µS**	6,03
Riesling	WKKL	LB +Metatartaric acid (17°C)	28 µS	23 µS	26 µS	**26 µS**	2,52
	Wabw	LB +Met (30° - 17°)	25 µS	21 µS	27 µS	**24 µS**	3,06
	W30	LB +Met (30°)	29 µS	33 µS	27 µS	**30 µS**	3,06

Codes*: abbreviation codes to be found in Table 71

Table 71 Abbreviation codes

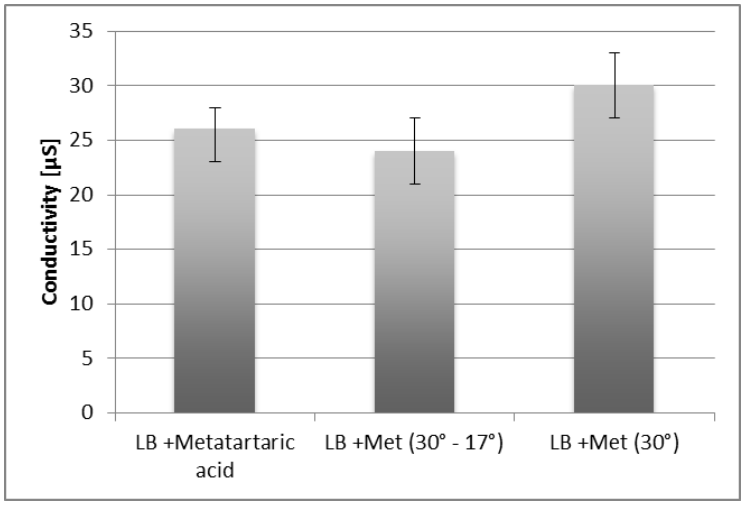
RKKL	Red wine - control cold (17°C) stored
Rabw	Red wine with alternate temperature
R30	Red wine only at 30°C
WKKL	White wine - control cold stored
Wabw	White wine with alternate temperature
W30	White wine only at 30°C

Figure 65 Bars chart of conductivity. Wine No. 10 Sangiovese –different temp.



Sangiovese (N:3)

Figure 66 Bars chart of conductivity in wine No. 9 –Riesling, different temperature



Riesling (N:3)

Following table has the knock down conductivity numbers followed by graphics that show the conductivity of different treatments on wines No. 12 to 16 of third trial, as shown in material and methods chapter.

One observation for Riesling wine when lysozyme is added is that this wine has the highest stability as well as the lowest pH.

Here on third trial and on second trial, all white wines where lysozyme is added became more stable because both agents act as colloid protectors (Brunner, 2013). Red wines turn less stable. When no lysozyme is added only metatartaric acid, both white and red become more stable. When lysozyme is added and followed by metatartaric acid results are similar to wines with only metatartaric acid.

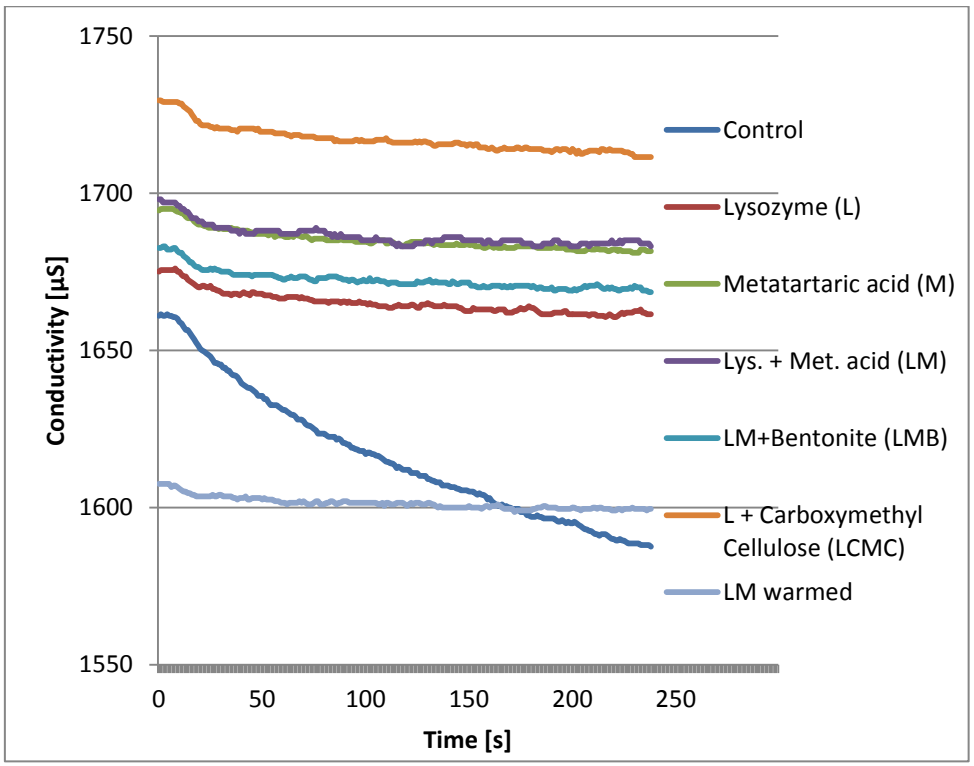
It would be interesting to know if bentonite changes the stability of wine when added after metatartaric acid in a future study.

Table 72 Knock down conductivity curves of wines 12 to 16

	Control	Lyso.	Met. Acid	Lys.+Met. acid	LM+Bentonite	L+CMC	LM warm
Mueller-Thurgau	74 µS	14 µS	13 µS	15 µS	14 µS	18 µS	11 µS
Riesling	100 µS	9 µS	7 µS	10 µS	9 µS	13 µS	10 µS
CS+Merlot	23 µS	36 µS	15 µS	17 µS	13 µS	44 µS	-
Dornfelder	45 µS	110 µS	15 µS	17 µS	14 µS	86 µS	-
Pinot Noir	89 µS	83 µS	11 µS	11 µS	35 µS	70 µS	-

All wines have the higher stability when treated with metatartaric acid as to be seen on following Figures: Figure 67 to Figure 71. Those graphics are representing Table 72. The interpretation is based on the difference between initial and final conductivity.

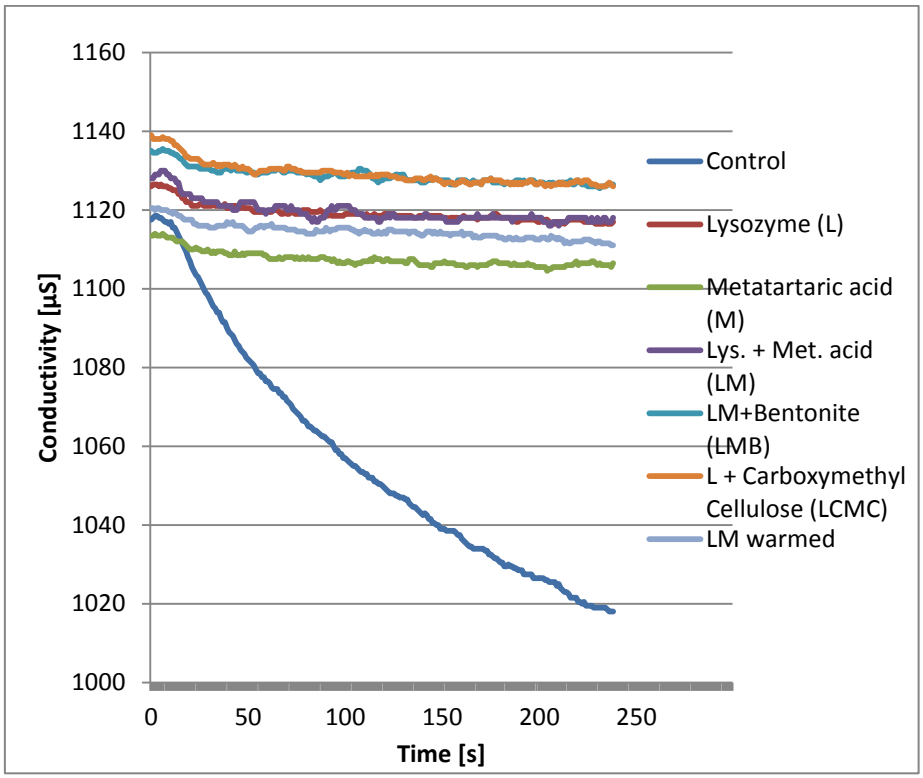
Figure 67 Figure Conductivity of Wine No. 12 Mueller-Thurgau



(Values given in mean n=3)

Riesling wine shown on Figure 68 has a fairly instable control if compared with all other samples. This can be seen on steep curve in medium blue colour.

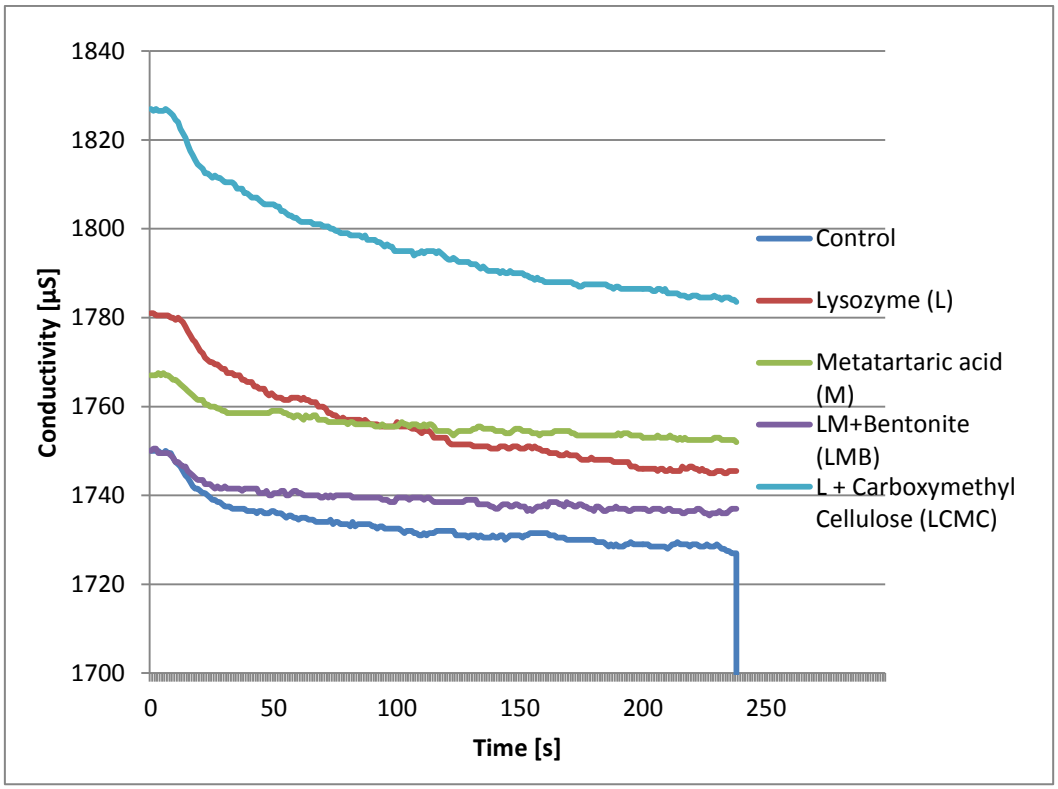
Figure 68 Conductivity of Wine No. 13 Riesling



(values given in mean n=3)

Cabernet Sauvignon and Merlot cuvée wine has similar difference values, CMC sample shows the greatest knock down.

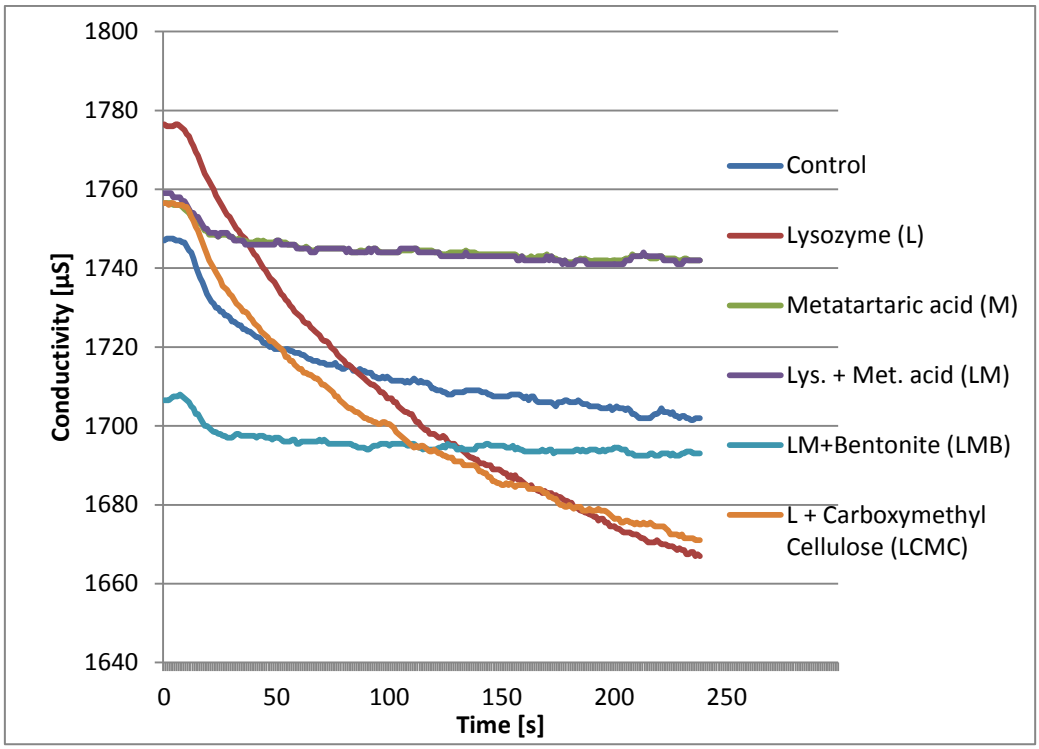
Figure 69 Conductivity of Wine No. 14 Cabernet Sauvignon + Merlot



(values given in mean n=3)

Dornfelder wine Figure 70 has an instable value for its control, L and CMC. Here as an explanation in red wines lysozyme and CMC reacts with phenols, as it has been discussed above in this work and presented on Figure 73 (Martin Vialatte, 2013). As an explanation for the raise in instability of wine after lysozyme addition is that the phenols that act as colloid protectors (Brunner *et al.*, 2013) are removed leaving the wine less stable. Further addition of metatartaric acid brings back wine tartaric stability.

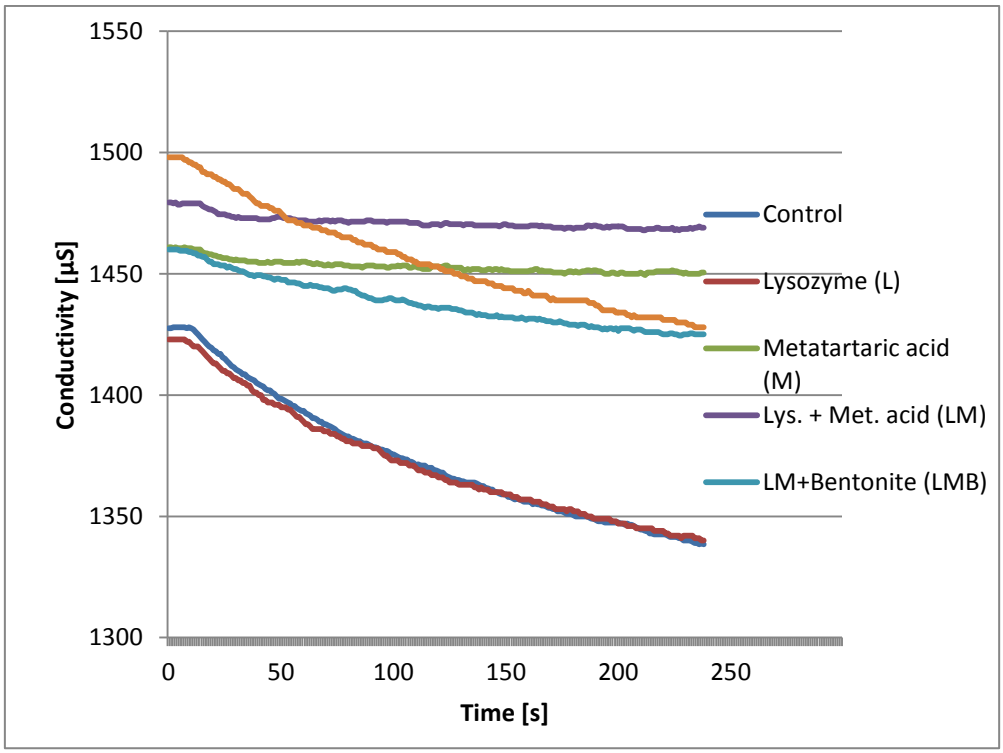
Figure 70 Wine No. 15 Dornfelder



(values given in mean n=3)

Finally the last graphic of conductivity shows Pinot Noir wine, which has unstable values 89 to 70 µS for control, lysozyme and slightly unstable for LCMC. All other samples are stable, being LM the most stable sample on the graphic, but with the same knock down number of 11 µS of conductivity, but graphic is very similar to M wine .

Figure 71 Conductivity of Wine No. 16 Pinot Noir



(values given in mean n=3)

5.10 Lysozyme protein removal with "Sartobind S"

This laboratory-scale-filter has been provided by the company *Sartorius* as an attempt of pre-trials to remove proteins from wine, i.e. as an attempt to quantify the amount reduced by filter, meaning another method of quantification.
These measures took place on 26th of August, on 02nd and 27th of September 2011 in the oenology department of the research centre of Geisenheim. The only wine to be analysed was a white Riesling – wine No. 9, due to the lower phenol content.

It is possible to detect that lysozyme has improved the stability of Riesling wine on Table 73. When bentonite is added the stability drops slightly back and metatartaric acid is added the sample is more stable than the control, but less stable than sample with only lysozyme added.

Table 73 Conductivity in µS of wines before **filtration with „Sartobind S"**

Wine	Sample	Measure 1	Measure 2	Measure 3	Mean	Deviation
Riesling	Control	37	40	41	39	2.08
Riesling	Lysozyme	15	16	15	15	0.58
Riesling	Lysozyme + Bentonite	17	17	17	17	0.00
Riesling	Lys. + Ben. + Metatartaric acid	31	33	30	31	1.53

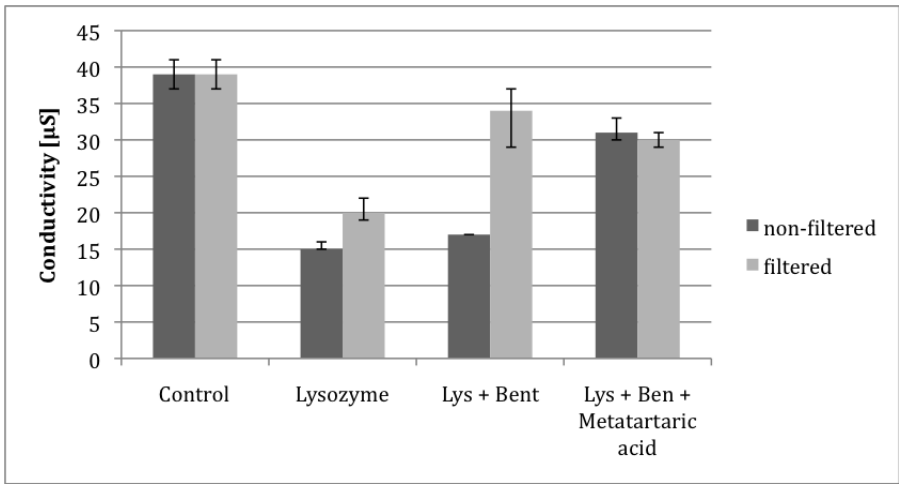
Table 74 Conductivity in µS of wines after **filtered with „Sartobind S"**

Wine	**Sample**	**Measure 1**	**Measure 2**	**Measure 3**	**Mean**	**Deviation**
Riesling	Control	37	41	39	39	2
Riesling	Lysozyme	22	19	20	20	1.53
Riesling	Lysozyme + Bentonite	29	35	37	34	4.16
Riesling	Lys. + Ben. + Metatartaric acid	31	31	29	30	1.15

On Figure 72 filtered and non-filtered wines are compared side to side. There is no difference for control wines. Wine treated with lysozyme and not filtered is more stable compared with filterer so did the wine treated with lysozyme and bentonite. Finally wines fined with LB and metatartaric acid show a slightly better stability when filtered.

This filter has the capacity of filtering/absorbing proteins and this case lysozyme, as we may see on Figure 75 and confirm on wine stability. As previously observed when lysozyme is added to Riesling wine its stability is better. Here when L wine is filtered the wine becomes less stable, because the level of lysozyme is lower. Nevertheless the reduction is really small and the use of an absorber filter in large scale would need improvements and would be probably of certainly high costs. Further research would be necessary.

Figure 72 Conductivity of wines before and after filtered with „Sartobind S"



(N:3)

5.11 Pictures of wine after-fining deposits

On Figure 73 left: Sangiovese treated with 50g/hL lysozyme after 24 hours (25L wine over 1.7L deposit with wine). On the right the wine separated of deposit after 6 days, resulting in a volume of over 1.2L of deposit. Van der Walls attractions are well known to occur between tannins and the non- polar regions of the proteins. Furthermore polyphenols bond to the surface of the proteins at one or more sites forming a monolayer that is less hydrophilic than the protein alone; this is followed by aggregation and precipitation (Ribéreau-Gayon *et al.*, 2006)

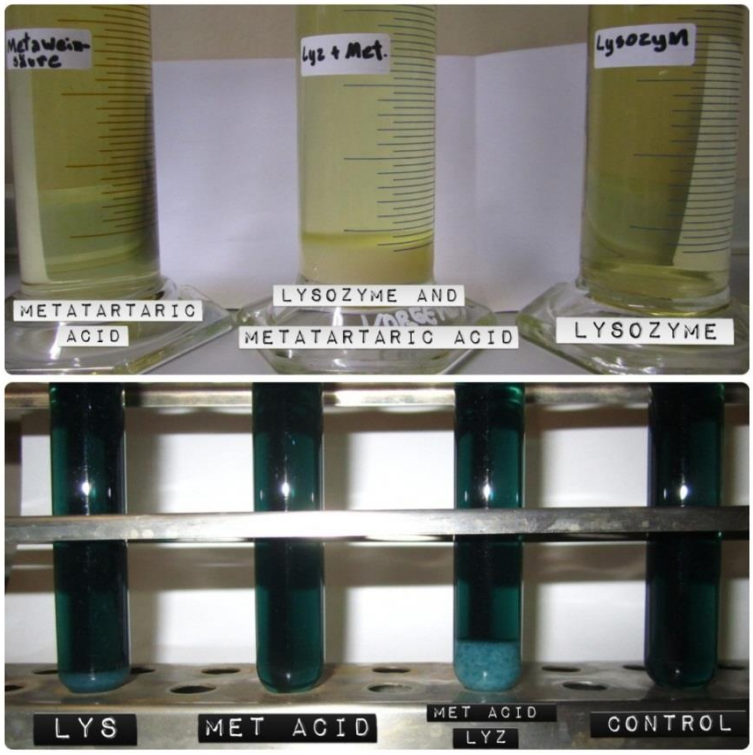
Figure 73 Lysozyme and phenols deposit – Sangiovese (second trial)

Figure 74 show a Riesling wine treated with lysozyme and further treated with metatartaric acid witch precipitate the protein.

Figure 74 Riesling treated with lysozyme & metatartaric acid – deposit (2nd trial)

Fining deposit visible on cylinder treated with both lysozyme and metatartaric acid together, while no deposit is found in wine fined only with lysozyme or only with metatartaric acid (Figure 74). Similar phenomenon can be seen on Figure 75; where a substance that precipitated proteins in suspension was added. A smaller deposit is found on wine treated with lysozyme, while a greater one is found on wine treated with both lysozyme and metatartaric acid.

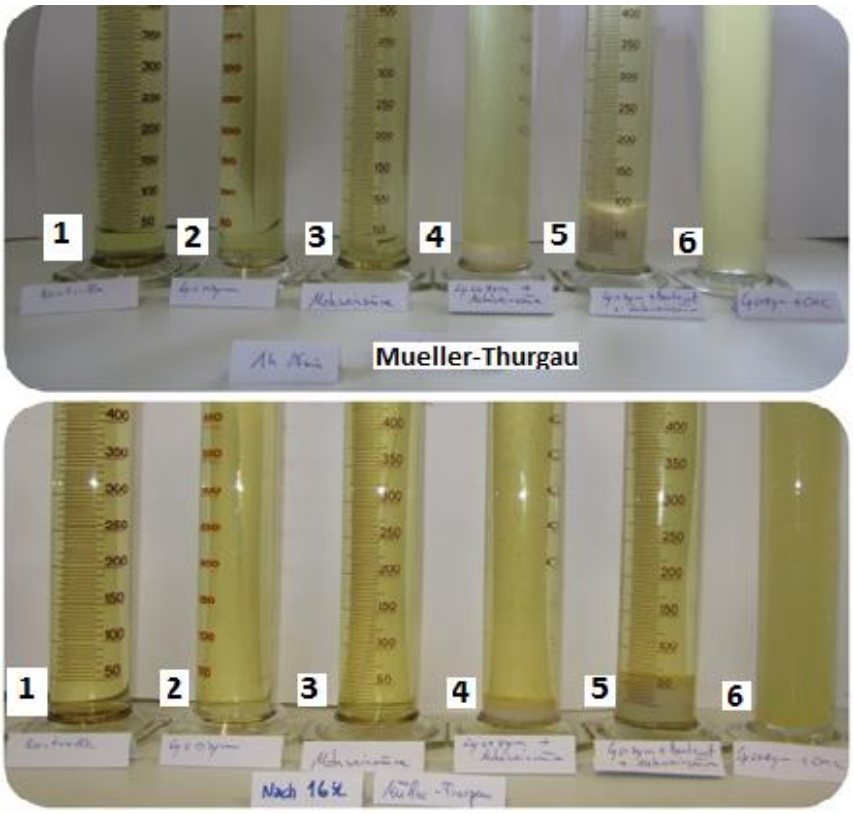
Figure 75 Pictures of fining deposits in white wine



The first two figures 76 and 77 are showing Mueller-Thurgau and Riesling wines, respectively. The top picture is taken less than two hours after fining while bottom one is after 16 hours. From the left to the right in Mueller-Thurgau and in Riesling pictures similar phenomena is observed:

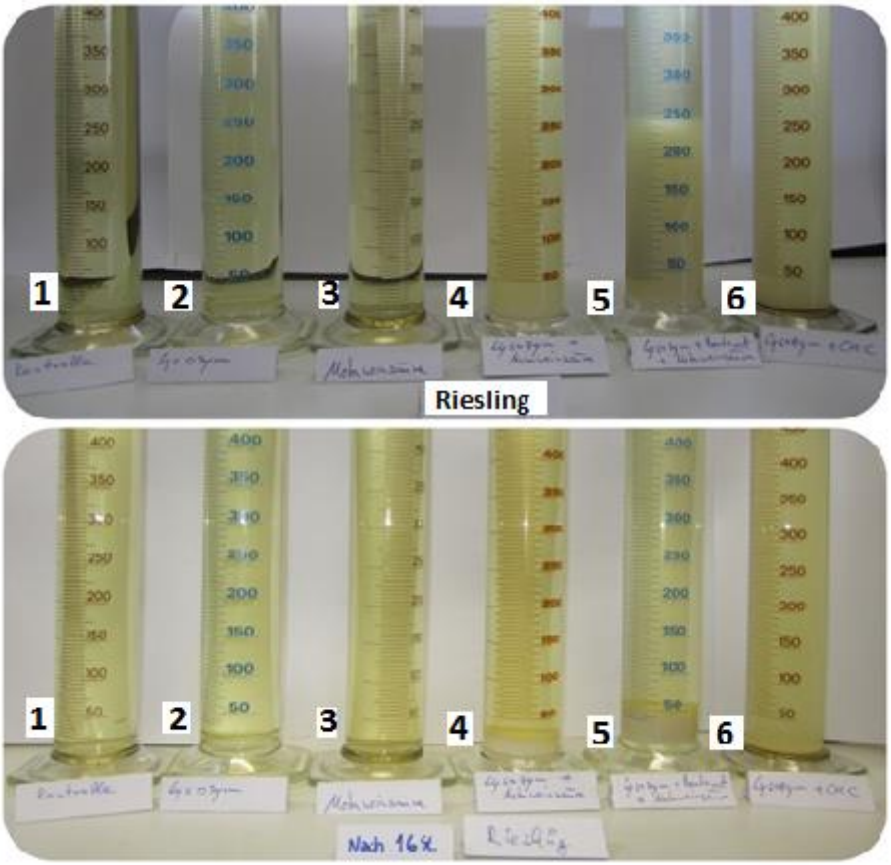
- 1 control and 3 metatartaric acid samples are clear and have negative signal for ELISA.
- 2 Lysozyme sample has an extremely fine deposit, almost invisible, by ELISA there is a positive signal of 151 ppm for Mueller-Thurgau and 129 ppm for Riesling.
- 4 Lys+Meta sample have a deposit of around 35mL and big particles in solution after 1h and 15 min and after 16h the deposit settles to half of it and there are still some particles in solution, but wine is less turbid than in the beginning. This precipitation meaning reduction, but still present in solution is confirmed by ELISA with 39 ppm for Mueller-Thurgau and 50 ppm for Riesling.
- 5 Lys+Bento+Meta have negative signal for both wines, here for the 4th time confirming previous studies
- 6 Lys+CMC sample is extremely turbid after 1h 15min but also after 16h, showing a very minor deposit. ELISA results are of 101 ppm for Mueller-Thurgau and of 123 ppm for Riesling, again showing good correlation of picture and ELISA results.

Figure 76 Lysozyme - third trial white wine Mueller-Thurgau



*1.Control, 2.Lysozyme, 3.Metatartaric acid, 4.Lys+Meta, 5.Lys+ Bentonite+ Meta, 6. Lys+CMC

Figure 77 Lysozyme - third trial white wine Riesling



*1.Control, 2.Lysozyme, 3.Metatartaric acid, 4.Lys+Meta, 5.Lys+ Bentonite+ Meta, 6. Lys+CMC

Figure 78 all red wines are together under each other after 2h and 15 min and as white wines these pictures can be well correlated to ELISA results. Sample number:

- 1 Control is clear and has no deposit in all wines
- 2 Lysozyme in Cab.Sav. wine has the greatest deposit of almost 100mL, the second is Dornfelder with almost 50mL and finally Pinot Noir with an unimportant deposit. These is directly correlated to ELISA results where Cab.Sav. has a reduction down to 8 ppm, Dornfelder down to 77 and Pinot Noir only down to 137 ppm. This is clearly explained by the phenol contend of wines, the higher the content the most dramatic the drop.
- 3 Metatartaric acid shows no deposit in all samples.
- 4 when lysozyme and metatartaric acid are used together there is a notable deposit in all three wines, they have all quite similar quantities, around 75 mL for Cab.Sav. and over 50 mL for Pinot Noir and Dornfelder. ELISA results are similar and follow Lysozyme sample trend: Cab.Sav. has the greatest reduction and Pinot Noir the lowest, but values here are closer: 6 and 20 ppm respectively.

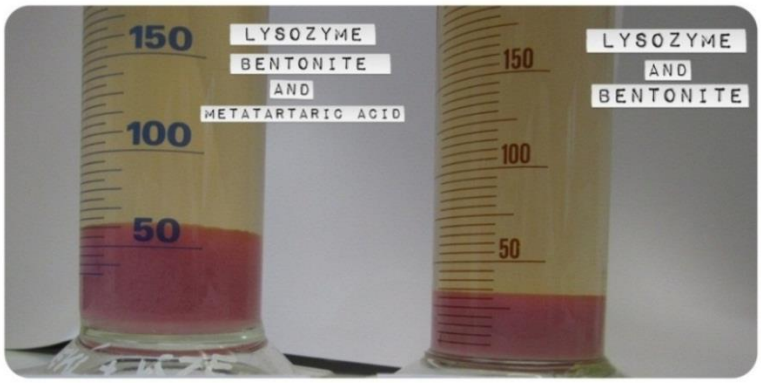
- 5 samples treated with lysozyme then bentonite and finally with metatartaric acid have one of the major deposits, again for Cab.Sav. one can observe the greatest of around 135mL and other wine just over 120mL. Results are equal – in none of the wines a positive. Meaning that all lysozyme is removed i.e. cannot be detected.
- 6 deposits here were around 200mL for Cab.Sav (to be seen on the picture there was floating particles on the top of the cylinder), around 80mL for Pinot Noir and a bit over 50mL for Dornfelder. Results are comparable for ELISA were Cab.Sav is reduced to 4 ppm, Dornfelder to 51 ppm and Pinot Noir to 56 ppm.

Figure 78 Lysozyme - third trial red wines

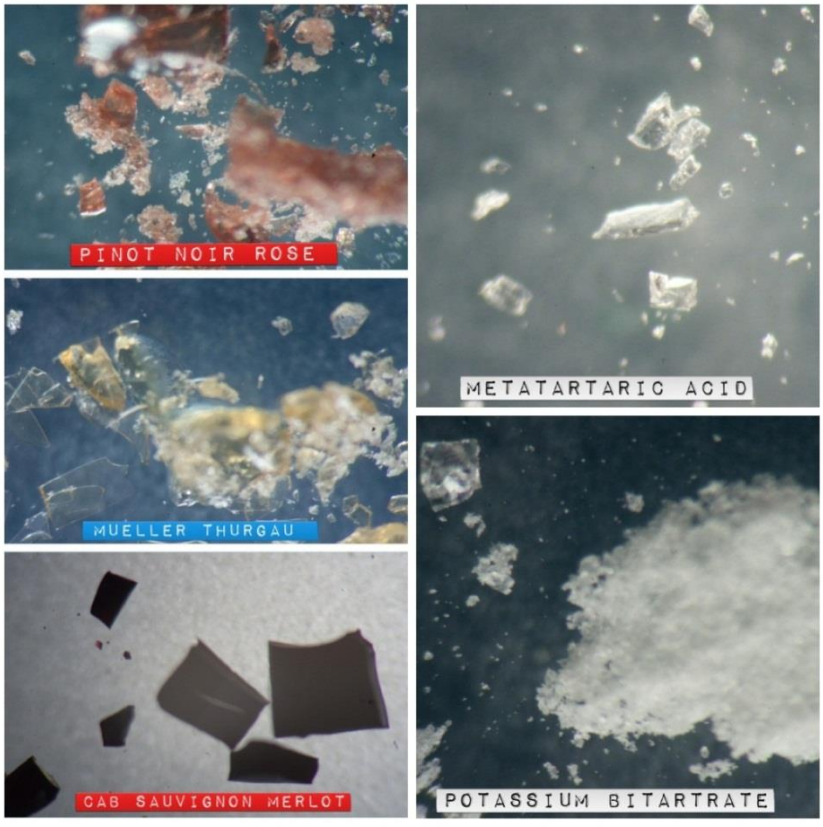
*1.Control, 2.Lysozyme, 3.Metatartaric acid, 4.Lys+Meta, 5.Lys+ Bentonite+ Meta, 6. Lys+CMC

The same phenomenon can be seen on rose wine, the deposit is greater when lysozyme and metatartaric acid are used in combination.

Figure 79 Picture of fining deposits on rosé wine



Following pictures are from, oven dried, wine deposits after fining process. All wines, in the first column of pictures, have been treated with lysozyme and metatartaric acid. They formed crystals that can be seen in pictures taken using microscopy. On the left there are pictures of metatartaric acid crystals as well as bitartrate of potassium. These pictures indicate the presence of metatartaric acid in wine deposit.



5.12 General wine analyses

The wines used for these studies were prompt fermented in the beginning of each trial and were provided by different wineries; mainly the research centre of Geisenheim. None of the wines were fined before, during or after fermentation. The following tables display all wines used during these studies with its analysis of conventional oenological parameters. Furthermore tables display region, variety and year.

General analysis of wines used on the first year

Table 75 First trial wines - No. 1 and 2

	1. Wine	2. Wine
Colour	White	Red
Variety	Mueller-Thurgau	Regent
Year	2009	2009
Region	Rheingau	Nahe
pH	3.4	3.6
Titratable acidity g/L (tart. acid)	4.5	5.3
Tartaric acid g/L	1.7	2.0
Malic acid g/L	0.3	0.2
Alcohol %v/v	12.0	12.3
Residue sugar g/L	1.3	2.4
Total dry extract g/L	19.5	23.5
Free/bound SO_2 mg/L	68/148	59/83
Relative density (20/20)	0.9918	0.9924
Refraction number	38.8	40.5
Total phenols mg/L	167	1240
Bentotest – Protein stability	Stable	Stable
Glycerine g/L	6.6	8.5
Volatile acidity g/L (acetic acid)	0.4	0.55
Wine code number FAG	0911	09082

General analysis of wines used on the second year

Table 76 Second trial wines - No. 3, 4 and 5

	3. Wine	4. Wine	5. Wine
Colour	White	White	White
Variety	Riesling	Blend	Mueller-Thurgau*
Year	2009	2009	2009
Region	Rheingau	Rheingau	Rheingau
pH	3.2	3.1	3.3
Titratable acidity g/L	9.7	9.3	7.5
Alcohol %v/v	12	12.5	12.4
Residue sugar g/L	3.8	5.3	0.3
Total dry extract g/L	26.5	30.5	22.5
Free/bound SO_2 mg/L	56/130	62/148	60/150
Relative density (20/20)	0.9949	0.9956	0.9938
Bentotest – Protein stability	stable	stable	Stable
Wine code number FAG	10730	10731	10732

Table 77 Second trial wines - No. 6, 7 and 8

	6. Wine	7. Wine	8. Wine
Colour	Red	Red	Red
Variety	Blend	Pinot Noir –Spätburgunder	Sangiovese
Year	2009	2009	2009
Region	Rheingau	Rheingau	Rheingau
pH	3.4	3.4	3.3
Titratable acidity g/L	6.1	6.6	6
Alcohol %v/v	12.4	14.2	13.6
Residue sugar g/L	1.2	1.0	0.5
Total dry extract g/L	25.7	28.1	26.3
Free/bound SO_2 mg/L	54/133	30/94	34/200
Relative density (20/20)	0.9938	0.9923	0.9931
Total phenols mg/L	1548	1126	2042
Bentotest – Protein stability	stable	stable	Stable
Wine code number FAG	10733	10734	10735

Table 78 Second trial wines - No. 9 and 10

	9. Wine	10. Wine
Colour	White	Red
Variety	Riesling	Sangiovese
Year	2009	2009
Region	Rheingau	Rheingau
pH	3.2	3.3
Titratable acidity g/L	10.6	6
Alcohol %v/v	12.1	13.4
Residue sugar g/L	25.2	4.0
Total dry extract g/L	55	26.5
Free/bound SO_2 mg/L	29/119	23/171
Relative density (20/20)	1.0055	0.9928
Refraction number	52.7	44.3
Total phenols mg/L	248	1924
Bentotest – Protein stability	Stable	Stable

General analysis of wines used on the third year

Table 79 Third trial wines - No. 11, 12 and 13

	11. Wine	12. Wine	13. Wine
Colour	White	White	White
Variety	Chardonnay	Mueller-Thurgau	Riesling
Year	2011	2011	2011
Region	Rheingau	Rheingau	Rheingau
pH	3.5	3.5	3.2
Titratable acidity g/L	7.7	7.3	7.4
Alcohol %v/v	12.4	13.2	13.7
Residue sugar g/L	4.2	2.5	9
Total dry extract g/L	25.2	33.9	32.8
Free/bound SO_2 mg/L	37/170	42/193	44/150
Relative density (20/20)	0.993	0.996	0.995
Refraction number	41.8	46.6	47.2
Total phenols mg/L	227	332	242
Bentotest – Protein stability	stable	stable	Stable
Wine code number FAG	2696	2697	2698

Table 80 Third trial wines No. 14; 15 and 16

	14. Wine	15. Wine	16. Wine
Colour	Red	Red	Red
Variety	Cabernet Sauvignon + Merlot (cuvée)	Dornfelder	Pinot Noir/ Spätburgunder
Year	2011	2011	2011
Region	Palatinate	Palatinate	Rheingau
pH	3.8	3.6	3.8
Titratable acidity g/L	5.2	5.3	4.6
Alcohol %v/v	12	12.2	13.8
Residue sugar g/L	3.5	4.3	10.6
Total dry extract g/L	25.1	26.9	32.6
Free/bound SO_2 mg/L	56/100	56/97	35/67
Reductones mg/L	57	50	33
Relative density (20/20)	0.994	0.994	0.995
Refraction number	41	42.1	47.2
Total phenols mg/L	1945	1915	1449
Protein stability	stable	stable	stable
Wine code number FAG	2699	2700	2701

6 Conclusion

Wines have been fined by means of natural fining agents over the centuries. Fining agents are known to precipitate and to be further removed by wine racking. Nevertheless not enough scientific research has been conducted on this topic and in recent years it has become quite important due to the allergic potential some of these fining agents proteins may present. The leading question of this work is if fining agent proteins remain in the wine; and if so, in what magnitude, and whether it could be threatening to an allergic consumer.

This study was carried out with the intention of determining by accurate methods of detection the existence of residues arising from fining agents used during the wine making process. The study is based on specific proteins that come from chicken egg albumen and from milk, i.e. ovalbumin, lysozyme and casein. This study also had the intention of testing whether other oenological methods were reasonable alternatives, in order to provide better approaches to reducing residues, as it is now officially required to declare any residue over 0.25 ppm on the label.

During the course of this study, and influenced by its collaboration, major changes in labelling law occurred. By approved analytical methods recommended by the OIV, it can be considered that casein, ovalbumin and lysozyme presence in the final wine for consumption is ‘detected’ just when the analytical values acquired are over than the detection limit value set at 0.25 mg/L. Only in these ‘detected’ cases is labelling obligatory. If these methods do not identify any protein from the food allergen in the wine, at that point it could be considered that no residue beyond the detection limit is present. In this way the industry could avoid overuse of precautionary labelling placing severe restrictions on dietary choices for consumers.

This study is a collaboration between three project partners, the University of Hamburg, Department of Chemistry, Institute of Food Chemistry – responsible for methods of detection; the Munich Technical University, Department of Dermatology and Allergology – responsible for allergy tests with allergic patients; and the Institute of Oenology of Geisenheim University – responsible for all wine making process and fining.

Selected detection method

The work completed in Hamburg, as part of this study, shows different ELISA methods that may be used for each wine, since this beverage has a notable matrix. A

commercially available testing kit for measuring protein residues was also used in this work. Methods are sensitive enough and followed the indication of OIV of LOD 0.25 ppm and LOQ 0.50 ppm.

Our Hamburg partner developed polyclonal antibodies from the immunization of host animals with the fining agents used in the cellar, which were used to develop diverse ELISA methods: indirect ELISA for casein (0.1 ppm LOD) and ovalbumin (LOD 0.006 ppm) can be used for white and low phenol red wines; indirect ELISA for lysozyme (LOD 0.006 ppm) for white and red wine; direct sandwich ELISA for ovalbumin in white and red wines (LOD 0.005 ppm); indirect sandwich ELISA for casein for white wine (LOD 0.01 ppm) and red wine (LOD 0.1-0.3 ppm).These different methods were aimed at achieving a higher specificity of polyclonal antibodies raised against every fining agent that was used for assay development in this study.

Ovalbumin is removed to a level below the detection threshold, even in worst case scenario when passed through any of following filtrations: Sterile filtration (⌀ 0.45 µm), polish filtration (⌀ 1 µm), membrane cartridge (⌀ 0.45 µm) and cross-flow filter. When white wine treated with the manufacturer's highest recommended dosage of ovalbumin was filtered with a diatomaceous earth filter, fine diatomaceous earth removed the ovalbumin to a level that showed negative in the ELISA test. However, when double the manufacturer's maximum recommended dosage was used in white wine, it showed a positive result of 0.36 ppm after diatomaceous filtration, this is 0.11 ppm higher than 0.25 ppm. On red wine all filtration methods brought the reading to not detectable signal, or to a non-detectable level.

Further methods used on red wines were effective at diminishing the ovalbumin to a non-detectable level. On white wines fined with ovalbumin further methods used do not show to be as efficient, only bentonite fining could remove ovalbumin protein to non-detectable level. Silica sol fining alone is not efficient neither is centrifugation, which is not truly capable of removing ovalbumin from colloidal solution by itself. When wines are flash-pasteurized, they show higher reading by ELISA than before the pasteurisation. This is probably related to protein conformation when heated, since it is thermo labile. Denaturing or partially denaturing treatments have an influence on immunochemical reactivity of tests such as ELISA. A rise in reactivity could lead to an over- or underestimation of the actual protein level as seen by previous studies and could be on this study the reason why the results are higher when wine is pasteurized (Rumbo *et al.,* 1996). A potential solution could be to filter the wine after fining and before heat treatment.

Both red and white wines fined with ovalbumin but not filtered frequently produced a negative ELISA signal, however, some had positive values up to 1.5 ppm. Winemakers wishing to bottle unfiltered wines should analyse the wines, to determine whether declaration is necessity.

Casein is removed from both red and white wines to not detectable levels by all methods of filtration used in this work. Further methods used are as efficient, apart from flash- pasteurization. But different to ovalbumin an additional sterile filtration decreases casein proteins to no longer being detectable. Just as for ovalbumin, the majority of red and white wines fined with casein already had a negative signal even without any treatments, however some wines in this work that were fined with casein and not given any other treatment did show low, but positive values, up to 1.3 ppm. Therefore, wines that have been fined with casein and not filtered may not automatically have negative signals, they should be tested, as they might need to carry a declaration.

In conclusion, when appropriate practices are followed for wines fined with ovalbumin or casein, there should be no detectable protein from the allergen left behind in the wine; however, if there is, labelling would still be required. Another possibility to avoid declaration is to use other proteins or alternative fining agents, as shown here before. On this work, and on further literature, vegetal proteins from pea and potato have similar and positive action on wines when compared with casein and ovalbumin.

Lysozyme

None of filtration methods used on the worst case study ware effective on diminishing lysozyme. The only treatment able to diminish lysozyme is further fining with bentonite followed by sterile filtration and only for the highest recommended dosage of lysozyme indicated by law (50g/hL). When the double the legal maximum of lysozyme was used not even bentonite was not capable to diminish it to low values. On supplementary studies accomplished using metatartaric acid and carboxymethyl cellulose (CMC), again the only way to completely remove lysozyme was by final addition of bentonite. Metatartaric acid precipitates lysozyme significantly, but the wines still always had a positive signal when tested with ELISA. In this study observation of precipitation shows that the main phenomenon diminishing lysozyme is due to the precipitation of matter, as the result of wine phenols or added fining. Lysozyme is a preservative and not a fining agent, therefore when used it must be declared, unless legally approved analyses are able to prove absence of this protein.

Clinic immunological tests

Although all patients recruited by Munich Technical University were confirmed to be assuredly allergic, the number of patients was in fact modest. From 14,530 candidates recruited by the University, based on their data base of allergic patients, only 7 patients actually took part in the clinical tests. None of them showed a positive reaction neither to skin prick test nor to the DBPC.

According to a recent study, the so called eliciting dose or the minimum amount necessary to start an allergic reaction is 0.1 mg for cow's milk and 0.03 mg for egg, and even this only produced a reaction in 1% of people who are known to be allergic (Allen, K. *et al*., 2014). According to these recently published numbers, if the 0.25 mg/L limit for allergens in wine is taken into account, one could theoretically say that a person included in Allen's study that is allergic to milk could drink the maximum of roughly 375mL of wine without getting an allergic reaction, while a person allergic to egg could drink only less than 125mL. In addition, it should be noted that this highly sensitive 1% of the allergic population presented in this study includes children. Skripak (2007) and Savage (2007) reported that 79% of children normally outgrow milk allergy and 68% egg allergy by age of 16, which means that less than 1% of allergic population are involved in wine consumption if related to Allen's study (Allen, K. *et al.*, 2014).

Winemaking methods are diverse around the world: it is a product that lacks standardisation due to its countless styles. These diverse styles are strongly influenced by the culture of every wine region or country.
It would be possible to describe specific manufacturing standards that would achieve wines with no allergenic potential. However, defining standards for winemaking could lead to an uninteresting and tedious final product. Diverse grape growing conditions, and the nature and desired features of the wines to be produced, lead to the use of different oenological practices. However producers those work in larger scale of wine might choose different ways to act than smaller producers.

There are better ways than creating manufacturing standards to produce wines that are safe for consumers and do not loose their identity. For example, using new technologies can avoid the use of certain allergenic fining proteins; and the use of allergen-free agents could be encouraged with incentives. Winemakers can also minimise the use of fining agents used to guarantee quality; or their use could simply be declared on the label.

Finally, it is essential that researches keep studying to bring new research into other intolerances and allergens to be found in wine.

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8 Annexes

Two bottles of Australian wines with fining agent declaration: “may contain milk products” and “this wine is clarified using milk products and traces may remain” due to Australia New Zealand Food Standards Code, where wine label declaration for milk and egg products is in vigour for some years.

Bottled wine labelled with a vintage date of 2002 or earlier

There are new allergen labelling requirements in the *Australia New Zealand Food Standards Code* (the Code) for all food including wine.

Despite these new allergen labelling requirements, bottled wine (including sparkling wine and fortified wine) labelled with a vintage date of 2002 or earlier will not have the presence of egg, fish, milk and nuts declared on the label.

Derivatives of egg, fish and milk may be used as fining agents in the wine production process. While these substances are largely removed through filtration, very small residue amounts may be present in the final product.

In addition, tannin derived from chestnuts is sometimes used as a wine additive.

These substances will not be declared on bottled wine labelled with a vintage date of 2002 or earlier, because bottled wines have a very long shelf life and can remain in circulation for many years and prior to the introduction of the Code in December 2002, manufacturers of wines were not required to label their presence.

Consequently, individuals who suffer from adverse reactions to egg, fish, milk and chestnuts should be aware that bottled wine (including sparkling wine and fortified wine) labelled with a vintage date of 2002 or earlier will not have these substances declared on the label, if present.

From 20 December 2004, bottled wine labelled with a vintage date of 2003 or later and all other alcoholic beverages must declare on the label derivatives of egg, fish, milk and nuts, when present.

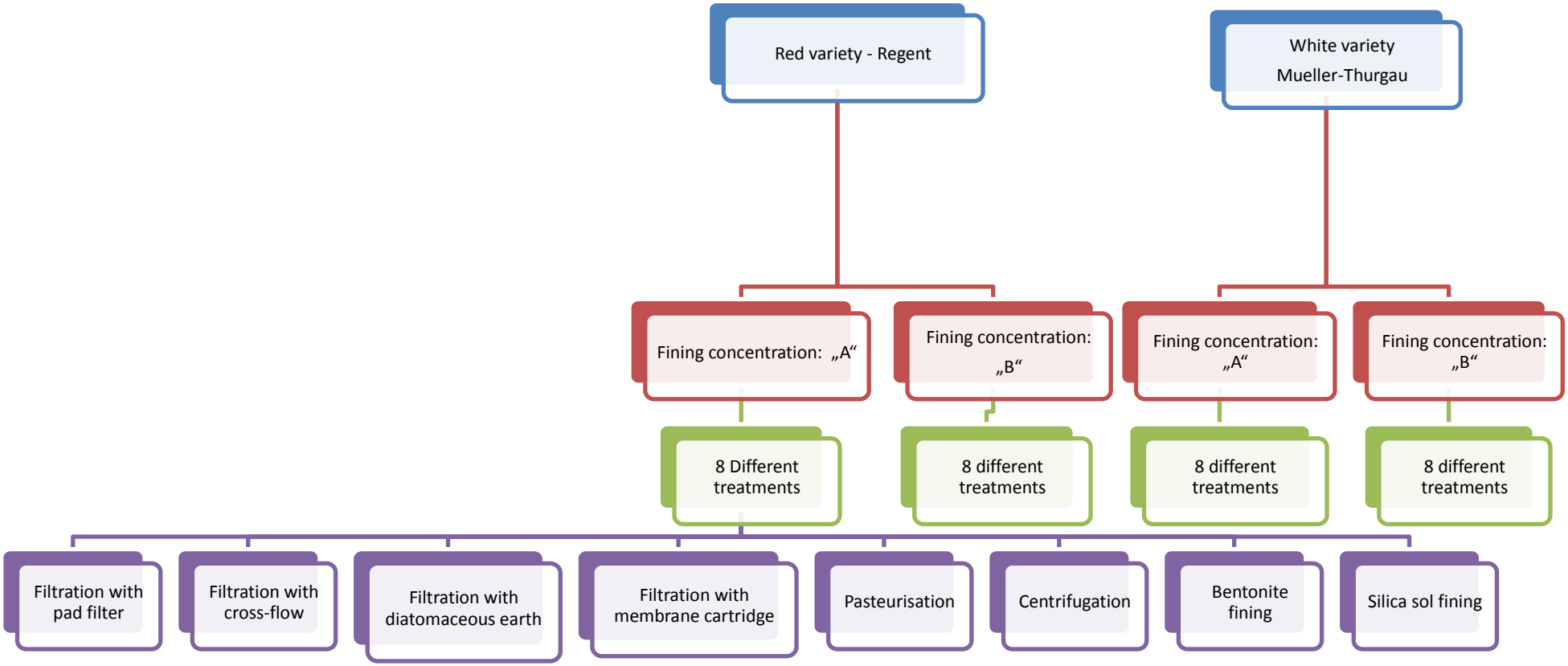
March 2004 (http://www.foodstandards.gov.au/scienceandeducation/factsheets/factsheets2004/bottledwinelabelledw2559.cfm**)**


SCAN TO LEARN MORE
750mL
WINE OF AUSTRALIA
12.5% ALC/VOL
1.5 STD DRINKS
Preservative (220) added. Produced with the aid of milk & egg products, traces may remain.
APPROX 7.4 STD DRINKS
Per 150ml
ENJOY WINE IN MODERATION


7.4
Standard Drin
750mL
12.5%
ALC/VOL
PRODUCED BY NEPENTHE WINES, JONES RD, BALHANNAH SA 5242
PRESERVATIVE (220) ADDED. MAY CONTAIN MILK PRODUCTS. WINE OF AUSTRALIA


PRODUCED BY
KINGSTON ESTATE WINES PTY LTD
STURT HIGHWAY, KINGSTON-ON-MURRAY
SOUTH AUSTRALIA 5331
THIS WINE IS CLARIFIED USING MILK PRODUCTS AND TRACES MAY REMAIN.
PRESERVATIVE (220) ADDED.
WINE OF AUSTRALIA
12.0% ALC/VOL
Standard Drinks
7.1
750mL

First Trial set up diagram



European Commission Implementing Regulation on wine labeling

L 171/4 EN Official Journal of the European Union 30.6.2012

COMMISSION IMPLEMENTING REGULATION (EU) No 579/2012

of 29 June 2012

amending Regulation (EC) No 607/2009 laying down certain detailed rules for the implementation of Council Regulation (EC) No 479/2008 as regards protected designations of origin and geographical indications, traditional terms, labelling and presentation of certain wine sector products

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Council Regulation (EC) No 1234/2007 of 22 October 2007 establishing a common organisation of agricultural markets and on specific provisions for certain agricultural products (Single CMO Regulation) [1], and in particular Article 121, first paragraph, point (m), in conjunction with Article 4 thereof,

Having regard to Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs [2] and in particular point (a) of the second subparagraph of Article 6(3a) thereof,

Whereas:

(1) For beverages containing more than 1,2 % by volume of alcohol, the first subparagraph of Article 6(3a) of Directive 2000/13/EC provides for the obligation to label all ingredients defined in paragraph 4(a) of that Article and listed in Annex IIIa to that Directive.

(2) The exemption from this obligation as regards wines, within the meaning of Annex XIb to Regulation (EC) No 1234/2007, placed on the market or labelled before 30 June 2012 until stocks are exhausted, as provided for in Commission Directive 2007/68/EC [3], as amended by Regulation (EU) No 1266/2010 [4], will no longer apply as from 30 June 2012.

(3) It is therefore necessary to establish detailed rules for labelling these beverages, including a mention of the substances referred to in Annex IIIa to Directive 2000/13/EC and used when making the beverages, if their presence can be detected in the final product using the analysis methods referred to in Article 120g of Regulation (EC) No 1234/2007 and if they consequently must be considered ingredients within the meaning of Article 6(4)(a) of Directive 2000/13/EC.

(4) In a multilingual context, labelling products using pictograms may improve the readability of the information provided to consumers and offer better guarantees for consumers. Therefore operators should be given the possibility of complementing written information with pictograms.

(5) Commission Regulation (EC) No 607/2009 [5] should therefore be amended accordingly.

(6) In order to prevent the new rules from affecting the marketing of products that are already labelled, it should be specified that they apply only to wines made completely or partially from grapes harvested in 2012 or later and labelled after 30 June 2012.

(7) The measures provided for in this Regulation are in accordance with the opinion of the Management Committee for the Common Organisation of Agricultural Markets,

HAS ADOPTED THIS REGULATION:

Article 1

Regulation (EC) No 607/2009 is amended as follows:

(1) Article 51 is replaced by the following:

'Article 51

Application of certain horizontal rules

1. For the purposes of indicating the ingredients as referred to in Article 6(3a) of Directive 2000/13/EC, the terms concerning sulphites/sulfites, milk and milk-based products and eggs and egg-based products that must be used are those listed in part A of Annex X.

2. The terms referred to in paragraph 1 may be accompanied, as applicable, by one of the pictograms shown in part B of Annex X.';

(2) Annex X is replaced by the contents of the Annex to this Regulation.

[1] OJ L 299, 16.11.2007, p. 1.
[2] OJ L 109, 6.5.2000, p. 29.
[3] OJ L 310, 28.11.2007, p. 11.
[4] OJ L 347, 31.12.2010, p. 27.
[5] OJ L 193, 24.7.2009, p. 60.

Article 2

This Regulation shall enter into force on the third day following that of its publication in the *Official Journal of the European Union*.

It is applicable, as regards the terms concerning milk and milk-based products and eggs and egg-based products referred to in Article 51(1) of Regulation (EC) No 607/2009, as amended by this Regulation, to the wines referred to in Annex XIb to Regulation (EC) No 1234/2007, made completely or partially from grapes harvested in 2012 or later and labelled after 30 June 2012.

Results for in vitro tests from Hamburg's ELISA

Tabelle 23: Messergebnisse der Ovalbumin-geschönten Weißweine (NWG: Nachweisgrenze, BG: Bestimmungsgrenze)

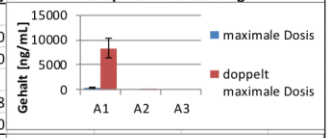
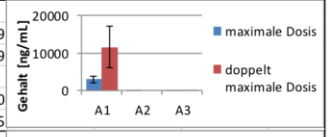
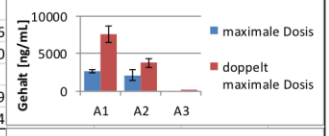
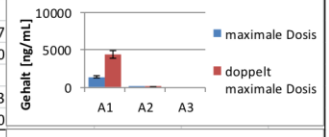
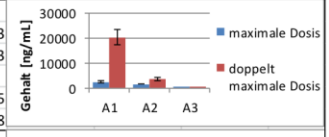
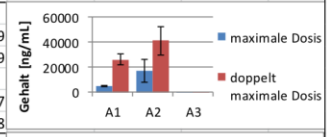
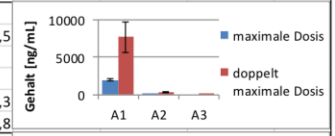
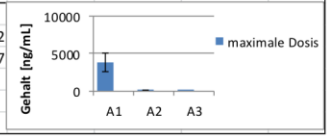
Wein	Stufe	Schönungsschritt	Gehalt [ng/mL]	CV [%] (n=9)	Abreicherungserfolg [%]	Graphische Darstellung
09915 EK-Filtration	A1	nach der Schönung	464,2	25,8		
	A2	nach K100-Filtration	NWG<x<BG	2,9	<100	
	A3	nach EK-Filtration	x<NWG	-	100,0	
09923 EK-Filtration	A1	nach der Schönung	8304,8	23,5		
	A2	nach K100-Filtration	18,4	63,0	99,8	
	A3	nach EK-Filtration	x<NWG	-	100,0	
09916 0,45µm-Filtration	A1	nach der Schönung	2952,3	26,2		
einfache Dosis	A2	nach 0,45µm-Filtration	62,4	9,6	97,9	
	A3	nach EK-Filtration	62,6	19,4	97,9	
09924 0,45µm-Filtration	A1	nach der Schönung	11531,7	47,9		
doppelte Dosis	A2	nach 0,45µm-Filtration	x<NWG	-	<100	
	A3	nach EK-Filtration	61,9	22,1	99,5	
09917 Zentrifugation u Schichten	A1	nach der Schönung	2635,2	7,6		
einfache Dosis	A2	nach Zentrifugation	2118,7	36,2	19,6	
	A3	nach EK-Filtration	NWG<x<BG	44,6	<100	
09925 Zentrifugation u Schichten	A1	nach der Schönung	7636,2	14,1		
doppelte Dosis	A2	nach Zentrifugation	3747,4	15,7	50,9	
	A3	nach EK-Filtration	49,6	37,9	99,4	
09918 Bentonit – Aktivit	A1	nach der Schönung	1404,6	9,3		
einfache Dosis	A2	nach Bentonit-Zugabe	31,9	18,1	97,7	
	A3	nach EK-Filtration	x<NWG	-	100,0	
09926 Bentonit – Aktivit	A1	nach der Schönung	4419,5	11,9		
doppelte Dosis	A2	nach Bentonit-Zugabe	120,9	18,6	97,3	
	A3	nach EK-Filtration	x<NWG	-	100,0	
09919 Kieselsol	A1	nach der Schönung	2485,6	14,1		
einfache Dosis	A2	nach Kieselsol-Zugabe	1608,1	6,8	35,3	
	A3	nach EK-Filtration	18,0	48,7	99,3	
09927 Kieselsol	A1	nach der Schönung	20286,8	14,6		
doppelte Dosis	A2	nach Kieselsol-Zugabe	3754,7	16,6	81,5	
	A3	nach EK-Filtration	49,7	26,9	99,8	
09920 Flash-Pasteurisation	A1	nach der Schönung	4729,2	12,4		
einfache Dosis	A2	nach Flash-Past.	16833,0	55,0	-255,9	
	A3	nach EK-Filtration	191,6	35,8	95,9	
09928 Flash-Pasteurisation	A1	nach der Schönung	25921,6	16,6		
doppelte Dosis	A2	nach Flash-Past.	41130,6	26,7	-58,7	
	A3	nach EK-Filtration	558,3	3,2	97,8	
09921 Feine Kieselgur	A1	nach der Schönung	1998,6	10,1		
einfache Dosis	A2	nach Kieselgur-Zugabe	90,7	15,5	95,5	
	A3	nach EK-Filtration	x=NWG	32,7		
09929 Feine Kieselgur	A1	nach der Schönung	7719,3	26,1		
doppelte Dosis	A2	nach Kieselgur-Zugabe	362,3	11,3	95,3	
	A3	nach EK-Filtration	14,2	21,9	99,8	
09922 Cross-Flow-Membran	A1	nach der Schönung	3814,4	33,8		
einfache Dosis	A2	nach Cross-Flow	105,3	48,6	97,2	
	A3	nach EK-Filtration	10,7	29,6	99,7	

Tabelle 24: Messergebnisse der Ovalbumin-geschönten Rotweine
(NWG: Nachweisgrenze, BG: Bestimmungsgrenze)

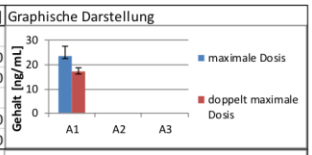
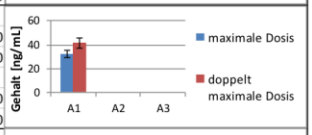
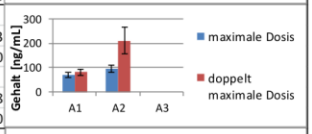
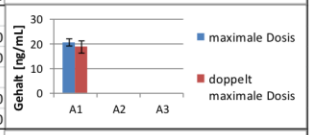
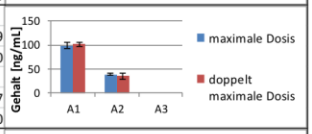
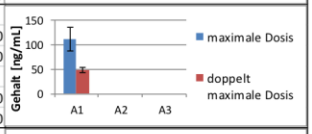
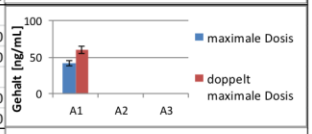
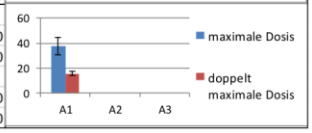
Wein	Stufe	Schönungsschritt	Gehalt [ng/mL]	CV [%]	Abreicherungserfolg [%]	Graphische Darstellung
09936 EK-Filtration	A1	nach der Schönung	23,6	17,3		
	A2	Nach K100-Filtration	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09944 EK-Filtration	A1	nach der Schönung	17,4	7,1		
	A2	Nach K100-Filtration	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09935 0,45µm-Filtration	A1	nach der Schönung	32,5	8,8		
	A2	Nach 0,45µm-Filtration	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09943 0,45µm-Filtration	A1	nach der Schönung	41,6	10,7		
	A2	Nach 0,45µm-Filtration	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09937 Zentrifugation u Schichten	A1	nach der Schönung	71,4	15,5		
	A2	nach der Zentrifugation	95,1	15,6	-33,3	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09945 Zentrifugation u Schichten	A1	nach der Schönung	81,9	14,4		
	A2	nach der Zentrifugation	211,1	26,0	-157,8	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09938 Bentonit - Aktivit	A1	nach der Schönung	20,8	7,3		
	A2	Nach Bentonit-Zugabe	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09946 Bentonit - Aktivit	A1	nach der Schönung	18,9	12,7		
	A2	Nach Bentonit-Zugabe	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09939 Kieselsol	A1	nach der Schönung	98,9	7,2		
	A2	nach der Kieselsol-Zugabe	38,7	5,7	60,9	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09947 Kieselsol	A1	nach der Schönung	102,0	4,4		
	A2	nach der Kieselsol-Zugabe	35,0	17,5	65,7	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09940 Flash-Pasteurisation	A1	nach der Schönung	111,4	22,4		
	A2	nach der Flash-Past.	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09948 Flash-Pasteurisation	A1	nach der Schönung	49,0	9,7		
	A2	nach der Flash-Past.	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09941 Feine Kieselgur	A1	nach der Schönung	41,6	8,9		
	A2	Nach Kieselgur-Zugabe	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09949 Feine Kieselgur	A1	nach der Schönung	60,3	8,2		
	A2	Nach Kieselgur-Zugabe	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09950 Cross-Flow-Membran	A1	nach der Schönung	37,6	18,3		
	A2	Nach Cross-Flow	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	
09942 Cross-Flow-Membran	A1	nach der Schönung	15,7	10,1		
	A2	Nach Cross-Flow	x<NWG	-	100,0	
	A3	Nach EK-Filtration	x<NWG	-	100,0	

Tabelle 25: Messergebnisse der Lysozym-geschönten Weißweine
(NWG: Nachweisgrenze, BG: Bestimmungsgrenze)

Wein	Stufe	Schönungsschritt	Gehalt [ng/mL]	CV [%] (n=3)	Abreicherungserfolg [%]	Graphische Darstellung
09915 EK-Filtration	L1	nach der Schönung	1633473,1	14,9		
einfache Dosis	L2	nach K100-Filtration	1474640,7	7,5	9,7	
	L3	nach EK-Filtration	1237114,8	13,9	24,3	
09923 EK-Filtration	L1	nach der Schönung	2583054,0	7,9		
doppelte Dosis	L2	nach K100-Filtration	2246517,4	4,7	13,0	
	L3	nach EK-Filtration	1661244,9	4,5	35,7	
09916 0,45µm-Filtration	L1	nach der Schönung	1647060,6	7,2		
einfache Dosis	L2	nach 0,45µm-Filtration	1391124,5	9,9	15,5	
	L3	nach EK-Filtration	1600196,6	14,8	2,8	
09924 0,45µm-Filtration	L1	nach der Schönung	2585224,2	4,7		
doppelte Dosis	L2	nach 0,45µm-Filtration	2378458,2	2,8	8,0	
	L3	nach EK-Filtration	2093030,3	7,4	19,0	
09917 Zentrifugation u Schichten	L1	nach der Schönung	1623034,8	8,0		
einfache Dosis	L2	nach Zentrifugation	1571579,4	7,0	3,2	
	L3	nach EK-Filtration	1436908,6	19,8	11,5	
09925 Zentrifugation u Schichten	L1	nach der Schönung	2274722,2	7,8		
doppelte Dosis	L2	nach Zentrifugation	2373015,7	4,2	-4,3	
	L3	nach EK-Filtration	2174654,2	1,9	4,4	
09918 Bentonit – Aktivit	L1	nach der Schönung	1545869,1	2,9		
einfache Dosis	L2	nach Bentonit-Zugabe	212811,7	10,8	86,2	
	L3	nach EK-Filtration	x<NWG	0,0	100,0	
09926 Bentonit – Aktivit	L1	nach der Schönung	2450479,7	3,6		
doppelte Dosis	L2	nach Bentonit-Zugabe	1278803,2	1,8	47,8	
	L3	nach EK-Filtration	1115536,1	4,1	54,5	
09919 Kieselsol	L1	nach der Schönung	2008958,7	6,3		
einfache Dosis	L2	nach Kieselsol-Zugabe	1821872,3	7,2	9,3	
	L3	nach EK-Filtration	1907038,3	10,9	5,1	
09927 Kieselsol	L1	nach der Schönung	2421786,6	6,4		
doppelte Dosis	L2	nach Kieselsol-Zugabe	2392481,8	12,0	1,2	
	L3	nach EK-Filtration	2084077,0	15,5	13,9	
09920 Flash-Pasteurisation	L1	nach der Schönung	1615974,7	5,2		
einfache Dosis	L2	nach Flash-Past.	1626860,7	4,4	-0,7	
	L3	nach EK-Filtration	1593492,0	15,5	1,4	
09928 Flash-Pasteurisation	L1	nach der Schönung	2696494,6	5,0		
doppelte Dosis	L2	nach Flash-Past.	2560631,1	11,9	5,0	
	L3	nach EK-Filtration	2064212,6	5,7	23,4	
09921 Feine Kieselgur	L1	nach der Schönung	1650410,5	3,3		
einfache Dosis	L2	nach Kieselgur-Zugabe	1568864,5	4,5	4,9	
	L3	nach EK-Filtration	1456775,2	12,5	11,7	
09929 Feine Kieselgur	L1	nach der Schönung	2657935,3	19,0		
doppelte Dosis	L2	nach Kieselgur-Zugabe	2637736,9	5,7	0,8	
	L3	nach EK-Filtration	2211123,6	4,9	16,8	
09922 Cross-Flow-Membran	L1	nach der Schönung	1375643,4	5,7		
einfache Dosis	L2	nach Cross-Flow	1556932,6	4,2	-13,2	
	L3	nach EK-Filtration	1217059,1	23,4	11,5	

Tabelle 26: Messergebnisse der Lysozym-geschönten Rotweine
(NWG: Nachweisgrenze, BG: Bestimmungsgrenze)

Wein	Stufe	Schönungsschritt	Gehalt [ng/mL]	CV [%] (n=3)	Abreicherungserfolg [%]	Graphische Darstellung
09935 EK-Filtration	L1	nach der Schönung	268908,9			
maximale Dosis	L2	nach K100-Filtration	212419,5		21,0	
	L3	nach EK-Filtration	169254,5		37,1	
09943 EK-Filtration	L1	nach der Schönung	517796,0			
doppelt maximale Dosis	L2	nach K100-Filtration	505262,3		2,4	
	L3	nach EK-Filtration	517678,9		0,0	
09936 0,45µm-Filtration	L1	nach der Schönung	332250,8			
einfache Dosis	L2	nach 0,45µm-Filtration	273096,5		17,8	
	L3	nach EK-Filtration	181846,7		45,3	
09944 0,45µm-Filtration	L1	nach der Schönung	573670,4			
doppelte Dosis	L2	nach 0,45µm-Filtration	595955,8		-3,9	
	L3	nach EK-Filtration	601959,0		-4,9	
09937 Zentrifugation u Schichten	L1	nach der Schönung	335882,1			
einfache Dosis	L2	nach Zentrifugation	287445,8		14,4	
	L3	nach EK-Filtration	191539,8		43,0	
09945 Zentrifugation u Schichten	L1	nach der Schönung	660410,5			
doppelte Dosis	L2	nach Zentrifugation	666267,3		-0,9	
	L3	nach EK-Filtration	670630,7		-1,5	
09938 Bentonit – Aktivit	L1	nach der Schönung	339249,7			
einfache Dosis	L2	nach Bentonit-Zugabe	62368,0		81,6	
	L3	nach EK-Filtration	x<NWG		100,0	
09946 Bentonit – Aktivit	L1	nach der Schönung	537270,1			
doppelte Dosis	L2	nach Bentonit-Zugabe	221731,9		58,7	
	L3	nach EK-Filtration	212302,4		60,5	
09939 Kieselsol	L1	nach der Schönung	311429,7			
einfache Dosis	L2	nach Kieselsol-Zugabe	232098,6		25,5	
	L3	nach EK-Filtration	172768,6		44,5	
09947 Kieselsol	L1	nach der Schönung	624654,4			
doppelte Dosis	L2	nach Kieselsol-Zugabe	574753,9		8,0	
	L3	nach EK-Filtration	588371,1		5,8	
09940 Flash-Pasteurisation	L1	nach der Schönung	247355,7			
einfache Dosis	L2	nach Flash-Past.	198304,5		19,8	
	L3	nach EK-Filtration	162489,8		34,3	
09948 Flash-Pasteurisation	L1	nach der Schönung	565734,4			
doppelte Dosis	L2	nach Flash-Past.	577799,5		-2,1	
	L3	nach EK-Filtration	585062,0		-3,4	
09941 Feine Kieselgur	L1	nach der Schönung	314123,8			
einfache Dosis	L2	nach Kieselgur-Zugabe	182491,0		41,9	
	L3	nach EK-Filtration	190514,9		39,4	
09949 Feine Kieselgur	L1	nach der Schönung	592383,1			
doppelte Dosis	L2	nach Kieselgur-Zugabe	492758,0		16,8	
	L3	nach EK-Filtration	539964,2		8,8	
09942 Cross-Flow-Membran	L1	nach der Schönung	172944,3			
einfache Dosis	L2	nach Cross-Flow	132649,1		23,3	
	L3	nach EK-Filtration	131741,3		23,8	
09950 Cross-Flow-Membran	L1	nach der Schönung	659122,0			
doppelte Dosis	L2	nach Cross-Flow	477852,3		27,5	
	L3	nach EK-Filtration	561136,7		14,9	

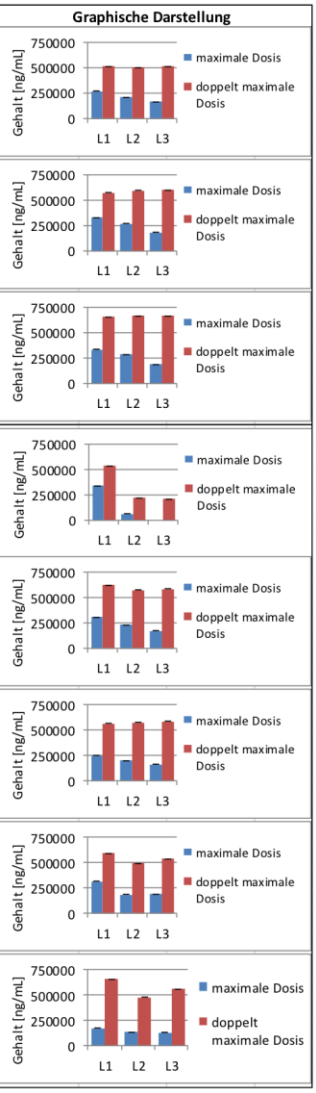


Tabelle 27: Messergebnisse der Kasein-geschönten Weißweine
(NWG: Nachweisgrenze, BG: Bestimmungsgrenze)

Wein	Stufe	Schönungsschritt	Gehalt [ng/mL]	CV [%] (n=9)	Abreicherungserfolg [%]	Graphische Darstellung
09915 EK-Filtration	K1	nach der Schönung	x<NWG	-		
einfache Dosis	K2	nach K100-Filtration	x<NWG	-		
	K3	nach EK-Filtration	x<NWG	-		
09923 EK-Filtration	K1	nach der Schönung	x<NWG	-		
doppelte Dosis	K2	nach K100-Filtration	x<NWG	-		
	K3	nach EK-Filtration	x<NWG	-		
09916 0,45µm-Filtration	K1	nach der Schönung	x<NWG	-		
einfache Dosis	K2	nach 0,45µm-Filtration	x<NWG	-		
	K3	nach EK-Filtration	x<NWG	-		
09924 0,45µm-Filtration	K1	nach der Schönung	x<NWG	-		
doppelte Dosis	K2	nach 0,45µm-Filtration	x<NWG	-		
	K3	nach EK-Filtration	x<NWG	-		
09917 Zentrifugation u Schichten	K1	nach der Schönung	x<NWG	-		
einfache Dosis	K2	nach Zentrifugation	x<NWG	-		
	K3	nach EK-Filtration	x<NWG	-		
09925 Zentrifugation u Schichten	K1	nach der Schönung	336,4	10,5		
doppelte Dosis	K2	nach Zentrifugation	x<NWG	-	100,0	
	K3	nach EK-Filtration	x<NWG	-	100,0	
09918 Bentonit – Aktivit	K1	nach der Schönung	363,9	5,6		
einfache Dosis	K2	nach Bentonit-Zugabe	x<NWG	-	100,0	
	K3	nach EK-Filtration	x<NWG	-	100,0	
09926 Bentonit – Aktivit	K1	nach der Schönung	NWG<x<BG	-		
doppelte Dosis	K2	nach Bentonit-Zugabe	x<NWG	-	100,0	
	K3	nach EK-Filtration	x<NWG	-	100	
09919 Kieselsol	K1	nach der Schönung	NWG<x<BG	-		
einfache Dosis	K2	nach Kieselsol-Zugabe	NWG<x<BG	-		
	K3	nach EK-Filtration	x<NWG	-	100	
09927 Kieselsol	K1	nach der Schönung	1353,5	43,8		
doppelte Dosis	K2	nach Kieselsol-Zugabe	NWG<x<BG	-	<100	
	K3	nach EK-Filtration	x<NWG	-	100	
09920 Flash-Pasteurisation	K1	nach der Schönung	x<NWG	-		
einfache Dosis	K2	nach Flash-Past.	2834,8	20,2		
	K3	nach EK-Filtration	x<NWG	-	100	
09928 Flash-Pasteurisation	K1	nach der Schönung	NWG<x<BG	-		
doppelte Dosis	K2	nach Flash-Past.	9484,6	36,2		
	K3	nach EK-Filtration	x<NWG	-	100	
09921 Feine Kieselgur	K1	nach der Schönung	NWG<x<BG	-		
einfache Dosis	K2	nach Kieselgur-Zugabe	x<NWG	-	100	
	K3	nach EK-Filtration	x<NWG	-	100	
09929 Feine Kieselgur	K1	nach der Schönung	892,3	5,7		
doppelte Dosis	K2	nach Kieselgur-Zugabe	x<NWG	-	100	
	K3	nach EK-Filtration	x<NWG	-	100	
09922 Cross-Flow-Membran	K1	nach der Schönung	x<NWG	3,8		
einfache Dosis	K2	nach Cross-Flow	x<NWG	-	<100	
	K3	nach EK-Filtration	x<NWG	-	100	

Recruitment of allergic subjects

Diagram showing the recruitment of allergic subjects done by project partner the Munich Technical University - Department of Dermatology and Allergology.